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CORRECTIONS

Volume 5, 1959

Page 656. Equation 3 should read

$$K = \frac{k_b T}{h} e^{\Delta S/R} - e^{-\Delta H/RT}.$$

Page 659. In Table II, E values for K_0 should all be minus quantities, i.e. $-4{,}000$; $-3{,}500$; and $-3{,}800$ cal/mole.

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MICROBIAL DECOMPOSITION OF HALOGENATED PROPIONIC AND ACETIC ACIDS[1]

P. HIRSCH[2] AND M. ALEXANDER

Abstract

Microorganisms actively degrading 2,2-dichloropropionic acid have been isolated and characterized. The responsible organisms, strains of *Nocardia* and *Pseudomonas*, liberate 90 to 100% of the halogen in dichloropropionate in periods of 3 weeks. Evidence for decomposition without chloride production has been obtained. Significant differences exist between isolates in the types of halogen-containing fatty acids utilized. Studies of the effect of halogen number and position show that beta-substituted propionates are more slowly attacked than the corresponding alpha-substituted compounds.

Introduction

The use of halogen-substituted fatty acids of the dichloropropionic, trichloropropionic, and trichloroacetic acid types as selective herbicides is continually increasing so that the fate of these compounds after application to soil is of great economic importance. A long period of persistence may be valuable for specific agricultural crops, but the phytotoxicity to succeeding crops may make a rapid disappearance desirable.

Little is known of the persistence of 2,2-dichloropropionic acid (dalapon) and trichloroacetic acid (TCA) in soil. Loustalot and Ferrer (7) demonstrated that warm and moist conditions favored the rapid disappearance of TCA while Thiegs (9) and Holstun and Loomis (3) showed the influence of temperature, moisture, and pH on the rate of dalapon detoxification. The transformations are largely or entirely biological since the compounds remained for long periods in sterile environments. Jensen (4, 5) and Magee and Colmer (8) have accomplished the isolation of several microorganisms active on dalapon, TCA, and related compounds, these isolates being strains of *Pseudomonas*, *Agrobacterium*, *Achromobacter*, and *Trichoderma*.

In view of the economic importance of the duration of activity of the fatty acid type pesticides in agricultural practice, a study was undertaken to ascertain the characteristics of some of the more active organisms involved in the decomposition.

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Materials and Methods

The basal salts solution for enrichment, isolation, and characterization of the bacteria contained 0.8 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 1.0 g $NaNO_3$, 0.01 g $FeSO_4.7H_2O$, 0.03 g $(NH_4)_2MoO_4$, 0.01 g $MnSO_4.4H_2O$, and 10 g $CaCO_3$ in 1.0 liter distilled water. Each of the halogenated fatty acids was neutralized, sterilized by filtration, and added to the autoclaved salts solution to a final concentration of 0.1%. In some experiments, yeast extract (0.05%) or casamino acids (0.05%) was included in the growth medium. Cultures were grown without agitation at 30° C in 250-ml Erlenmeyer flasks containing 100 ml of medium with the $CaCO_3$ sterilized separately. Three to five replicates were used in all experiments.

Unlabelled and C^{14}-labelled 2,2-dichloropropionate and untagged 2,2,3-trichloropropionate were kindly provided by the Dow Chemical Company, Midland, Mich., 2-iodo-, 2-bromo-, and 2-chloro-propionic acids and the monofluoro- and tribromo-acetic acids were obtained from K and K Laboratories, Jamaica, N.Y., while the other chemicals were obtained from Eastman Kodak Company, Rochester, N.Y.

Activity was determined by measurement of halide release using $AgNO_3$ and $KCrO_4$ (10) or by visual estimation of growth, the formation of acid, or the decrease in substrate concentration. In the latter procedure, used only for dichloropropionate, the substrate level was determined by (*a*) titrimetric assay of the chloride liberated through autoclaving for 50 minutes in the presence of $NaHCO_3$ and (*b*) measuring the amount of pyruvic acid released following the autoclaving procedure by the color produced with dinitrophenylhydrazine. All halide values reported are corrected for inorganic halide found in uninoculated controls.

Results

Serial dilutions prepared from soils incubated with 0.025% 2,2-dichloropropionate or trichloroacetate were streaked upon the basal agar medium containing 0.1 to 0.3% of the respective chemical. Following incubation and subsequent purification on the same media, pure cultures of a number of bacteria, *Streptomyces*, *Nocardia*, *Penicillium*, and *Aspergillus* spp. were obtained. All but one, an actinomycete, produced no chloride when tested in liquid culture containing 0.1% of the halogenated substrate regardless of the presence or absence of yeast extract and casamino acids. Similar observations were made by Magee and Colmer (8), who isolated a diverse group of bacteria, actinomycetes, and fungi which grew in the presence of dichloropropionate but failed to liberate chloride from it. A number of oligocarbophilic *Nocardia* strains, organisms capable of heterotrophic growth in organic matter-free, inorganic salts solution, developed readily in the media containing 0.1% dichloropropionate or trichloroacetate, but no ionic chloride in excess of the controls was released in 14 days. Many organisms thus grew rapidly in the presence of high concentrations of these two herbicidal compounds. The active

actinomycete produced little or no chloride in unsupplemented dichloropropionate media or in the presence of casamino acids, but 71 p.p.m. chloride were found after 16 days' incubation in solutions containing 0.05% yeast extract.

Subsequent isolates were obtained by inoculating soils initially incubated with chlorinated pesticides (0.025 to 0.050%) into the basal medium modified to contain the specific herbicide at a concentration of 0.1%. Such soil enrichments are quite active in dehalogenation (Table I). In several of these enrichments, all the organic chlorine was liberated in periods of 9 days. Transfers from these initial mixed populations carried through the same medium were even more active, and complete halide release from 0.1% dichloropropionate in the presence of yeast extract required less than four days.

TABLE I

Dichloropropionate and trichloroacetate decomposition in soil enrichment cultures

Enrichment No.	Soil amendment	C source in medium	Days			
			3	5	7	9
			Chloride released, p.p.m.			
1	TCA	TCA	28	46	128	328
2	TCA	TCA	50	80	174	390
3	TCA	TCA	36	46	139	224
4	Dichloropropionate	Dichloropropionate	50	53	213	375
5	2,4-D*	Dichloropropionate	28	32	375	540

*2,4-dichlorophenoxyacetic acid.

Aliquots from the more active mixed cultures were streaked upon agar containing the respective substrates as sole carbon sources, and the resultant colonies were purified by use of herbicide and nutrient agar. The dichloropropionate isolates remained viable and active when maintained on sterile, dry soil for periods of at least 6 months, but the trichloroacetate organisms readily lost their activity in one to two serial transfers on solid media. The addition of yeast extract, casamino acids, soil extract, or additional calcium carbonate did not alter this declining capacity. Similar difficulties have been reported for cultures of *Arthrobacter globiforme* utilizing 2,4-dichlorophenoxyacetic acid (2). No further investigations were made of the TCA-decomposing isolates.

Most of the strains active upon dichloropropionate fell into a few separate types so that only eight of the isolates were further characterized to establish their identity. Three strains active on the halogenated propionic acid were short, Gram-negative rods, 0.4–0.8×0.5–2.8 μ, occurring singly or in short chains. Growth was rapid in purple broth base (Difco) containing 0.5% arabinose, glucose, maltose, lactose, sucrose, glycerol, or mannitol, but neither acid nor gas was formed except on the latter carbohydrate where slight acidity was developed. Two of three strains made litmus milk alkaline while one produced an acid reaction. No liquefaction of gelatin could be detected. All three organisms developed well on nutrient or dichloropropionate agar, the colonies being

flat and glistening with a beige to yellow coloration. Growth in liquid was accompanied by an increase in turbidity followed by the appearance of a thin, glistening pellicle. The organisms were all motile, and electron micrographs showed the presence of a long, single, polar flagellum. The general characteristics of these three strains suggest that they are members of the genus *Pseudomonas*.

The second group of five isolates were Gram-positive, and a weak acid-fastness was evidenced in several media. Growth in liquid culture was either in the form of a surface pellicle or as the thin, spreading, surface growth typical of certain alpha-nocardias. Colonies on agar were yellow to orange, up to 6 mm in diameter, raised, and having a small central invagination. The surface of the colonies was granular, dull, and moist with the edges showing a definite filamentous pattern. On potato plugs, a rudimentary mycelium was formed. Young cultures frequently produced branched hyphae, and this true mycelium readily divided into short rods and coccoidal bodies. Growth was rapid upon nutrient agar, dichloropropionate agar, glycerol-glycine agar, potato slices, and in litmus milk, but no soluble pigments were noted. Growth likewise occurred in media containing arabinose, glucose, maltose, lactose, sucrose, or glycerol, but neither acid nor gas was liberated with the exception of a single strain which acidified the glucose broth. All isolates produced acid but no gas from mannitol. An alkaline reaction was developed in litmus milk at the end of 2 weeks, but gelatin was not liquefied. One of the organisms exhibited weak motility, and the branched cells grown on nutrient agar possessed a consistent capacity for movement. The existence of the true mycelium during the early phases of growth, the staining characteristics, the observation of a rudimentary aerial mycelium, and various culture traits suggest the placement of these five organisms in the genus *Nocardia*.

The activity of various isolates characterized as *Pseudomonas* or *Nocardia* spp. was determined in a dichloropropionate medium containing yeast extract and $CaCO_3$ (Table II). The rate of decomposition is expressed as p.p.m. chloride released, and the theoretical halide liberation was ascertained by

TABLE II

Halide release by strains of dichloropropionate metabolizing microorganisms

Strain	Days				% theoretical Cl release	Final pH*
	1	4	9	21		
	Chloride, p.p.m.					
Pseudomonas 395	0	509	—	718	97.2	7.1
Pseudomonas 396	0	518	—	720	97.3	6.9
Pseudomonas 409	0	541	535	734	99.2	6.9
Nocardia 398	0	532	556	659	89.1	6.8
Nocardia 399	0	344	566	734	99.2	6.9
Nocardia 400	0	516	587	771	104.2	6.9
Nocardia 401	0	308	545	704	95.1	7.0
Nocardia 414	0	319	570	771	104.2	6.8

*Initial pH, 7.3.

autoclaving control flasks made alkaline prior to heating. The results demonstrate that the bacteria were generally more active than the actinomycetes, but there were appreciable differences in rates of dehalogenation among the various isolates. From 89 to 100% of the organic halogen was released in a period of 3 weeks by all isolates. In the absence of yeast extract, the three pseudomonads and the five nocardias grew, and the chloride release was almost quantitative, but the rate was typically slow. Because of the stimulatory effect of the yeast extract, an experiment was set up to examine the influence of different concentrations. The results summarized in Table III demonstrate that yeast extract affects the rate but not magnitude of decomposition whereas $CaCO_3$ influences the net chloride production, an effect probably associated with acid accumulation.

TABLE III

Influence of yeast extract and calcium carbonate on dichloropropionate decomposition

		Chloride produced, p.p.m.					
		0.2% yeast ext.		0.1% yeast ext.		0.0% yeast ext.	
Strain	Days	$CaCO_3$	$-CaCO_3$	$CaCO_3$	$-CaCO_3$	$CaCO_3$	$-CaCO_3$
Nocardia 398	2	143	104	64	44	30	3
	7	542	531	503	446	503	426
Pseudomonas 409	2	258	248	79	102	18	22
	7	530	395	497	405	501	385

Two general mechanisms of attack on the dichloropropionate molecule may be considered, one involving both a dehalogenation and an oxidation of the resultant three-carbon moiety and a second in which there is no ionic chloride released. The five actinomycetes and the three pseudomonads are examples of the former generalized sequence. In one study, for example, *Nocardia* 398 was cultivated for 9 days at 30° C in a sealed desiccator in a medium having 0.1% yeast extract and 1000 p.p.m. of 2,2-dichloropropionate containing 22,200 c.p.m. of 2,2-dichloropropionate-2-C^{14}. The carbon dioxide respired was trapped in $Ba(OH)_2$, washed, dried, and counted with a thin window GM-tube. The medium was acidified, the surface growth harvested, washed with acidified water and distilled water, dried, and the radioactivity similarly counted. From the herbicide supplied, an average of 513 p.p.m. chloride was released as the free halide, i.e., both halogens were liberated from the alpha carbon. The alpha carbon itself was found in both cells and CO_2, 6628 c.p.m. above background being recovered in the cellular material and 6230 c.p.m. in the CO_2. Consequently, it is apparent that this organism is capable of attacking the alpha carbon and of totally dehalogenating the molecule.

Evidence for the second mechanism was obtained in a study in which microbial activity was measured not only by the appearance of free chloride but also by determining the residual concentration of carbonaceous substrate. Cultures of an active pseudomonad and of apparently inactive actinomycetes and a penicillium were inoculated into the basal medium modified to contain

0.01% dichloropropionate and incubated for 11 days at 30° C. The results of Table IV show that *Pseudomonas* 395 likewise liberated all of the organic chlorine. It is interesting, however, that one streptomycete could attack the pesticide and bring about the oxidation of 11.6% of the quantity supplied, yet no significant amount of ionic Cl was released. For most active cultures, however, halide titrations were adequate as an indication of decomposition.

TABLE IV

Microbial decomposition of dichloropropionate measured by halide production and substrate disappearance

Strain	% theoretical Cl release	Dichloropropionate, p.p.m.	
		Added	Recovered*
Pseudomonas 395	100	100	2.8
Streptomyces 411	2.7	100	88.4
Streptomyces 382	2.7	100	96.5
Streptomyces 268	0.0	100	96.0
Nocardia 100	0.0	100	97.2
Penicillium sp.	0.0	100	100.4

*Recovery measured after 11 days.

A number of halogenated fatty acids in addition to 2,2-dichloropropionic acid have become of importance in recent years for the control of weeds in agronomic crops. It was therefore of considerable interest to determine the influence of position and type of halogen substitution on the availability of substituted aliphatic acids to microbial attack. Only the propionate and acetate series were considered since these at present are of greatest agricultural significance. Each compound was added following neutralization to 100 ml of yeast extract – basal salts solution contained in 500-ml Erlenmeyer flasks to give a final concentration of 0.1%. *Pseudomonas* 409 and *Nocardia* 398 were used as test organisms to represent the two taxonomic groups isolated. After inoculation, aliquots were removed at regular intervals for titrimetric determinations, and the results presented in Tables V and VI are corrected for halide release in uninoculated controls. Notwithstanding the different growth rates of the two organisms, it is immediately apparent that definite differences exist. For example, the actinomycete actively dehalogenated iodoacetate, a compound not utilized by the bacterium, whereas the pseudomonad but not the nocardia was capable of utilizing trichloroacetate. With the exception of fluoroacetate, a compound not attacked by either organism, the *Nocardia* sp. was active on all monohalogenated acetate derivatives while the pseudomonad utilized such compounds with difficulty or not at all. The resistance of fluoroacetate may be in some way related to its role as an inhibitor in the tricarboxylic acid cycle.

Several areas of similarity are likewise readily apparent. Both organisms were active on 2,2-dichloropropionate and on the 2-chloro-, 2-bromo-, and 2-iodo-propionate, but the alpha-dichloro fatty acids persisted for shorter periods than the corresponding alpha-monochloro compounds. In each instance, the alpha-halogenated propionate was metabolized at a greater rate

than its corresponding acetate. Neither isolate degraded the fluorinated compounds, but growth occurred in their presence at the expense of the yeast extract so that the apparent resistance of the organic acid is not the result of a bacteriostatic or bactericidal action. Of the mono-halogenated propionates, the beta-substituted molecule possessed greater resistance than the alpha form; this is in agreement with the inability of either strain to dehalogenate the

TABLE V

Metabolism of halogenated fatty acids by *Nocardia* 398

	Days					
	2	4	6	9	11	20
Compound	Halide released, p.p.m.					
2-Chloropropionate	163	343		340		370
2-Bromopropionate	170	432		400		409
2-Iodopropionate		576				590
3-Chloropropionate	53	208		260		
3-Bromopropionate		0		0		0
3-Iodopropionate	0	0		25		
2,2-Dichloropropionate	83	520		570		
2,3-Dibromopropionate	0	0		0		0
2,2,3-Trichloropropionate			0		0	
Fluoroacetate	0	0		0		0
Chloroacetate	50		81		98	121
Bromoacetate	120		303		420	
Iodoacetate		165		304		394
Dichloroacetate	1	53		143		
Trifluoroacetate	0		0		0	0
Trichloroacetate	0		0		0	0
Tribromoacetate		22		40		56

TABLE VI

Metabolism of halogenated fatty acids by *Pseudomonas* 409

	Days					
	2	4	6	9	11	20
Compound	Halide released, p.p.m.					
2-Chloropropionate	17	154		321		378
2-Bromopropionate	22	180		388		390
2-Iodopropionate		141				277
3-Chloropropionate	10	9		53		
3-Bromopropionate		0		0		
3-Iodopropionate	0	0		23		
2,2-Dichloropropionate	98	490		536		
2,3-Dibromopropionate	0	0		0		
2,2,3-Trichloropropionate			0		1	
Fluoroacetate	0			0		0
Chloroacetate	0		11		39	56
Bromoacetate	8		26		20	
Iodoacetate		0				0
Dichloroacetate	1	80		180		
Trifluoroacetate	0		0		0	
Trichloroacetate	5		59		105	
Tribromoacetate		53		54		79

2,3-dibromo- and the 2,2,3-trichloro-propionate. The results therefore suggest, for these strains at least, that beta-substituted propionates are not suitable substrates, an observation which may have some bearing upon the persistence of such herbicides in soil.

Discussion

Many of the major herbicides used in agricultural practice, in turf management, or for the control of weeds along highway right of ways disappear with time, and this lack of persistence is usually the result of microbiological activities. Yet, with the exception of a small number of these pesticidal chemicals, final proof of the biological nature of the detoxification or knowledge of the specific microorganisms concerned is lacking. With dichloropropionate and trichloroacetate, the two most important halogen-containing fatty acid herbicides, evidence is available that the short persistence and the decomposition in field soil is the result of the activity of some segment of the natural microflora (3, 7).

A number of microorganisms have been shown to be capable of growth upon media containing as their sole carbon sources several chlorinated acetic and propionic acids. The compounds studied included several agriculturally important herbicides and chemicals related structurally to them. In addition to the pseudomonads and nocardias isolated herein, several other genera of bacteria and fungi have been shown to utilize alpha-chlorine-substituted acetic and propionic acids, these including *Pseudomonas*, *Agrobacterium*, *Arthrobacter*, *Achromobacter*, *Trichoderma*, *Penicillium*, and *Clonostachys* (4, 5, 6, 8). The observation that one of the present *Nocardia* isolates is motile is of interest because motility is considered to be relatively rare in the genus.

Several conclusions may be drawn on the basis of the ability of the *Pseudomonas* sp. and *Nocardia* sp. to utilize halogenated fatty acids. Both microorganisms utilized the four alpha-halogenated propionates provided, and the rate of decomposition was characteristically greater for the alpha-halogenated compound than for the corresponding beta derivative so that it seems that a chlorine on the number three carbon atom retards enzymatic attack. Differing from the alpha-halogenated propionates, all of which were metabolized, the bacterium acted on the tribromo- and trichloro-acetates but not the trifluoroacetate while the actinomycete dehalogenated only the brominated compound. It is worthy of note that organisms utilizing 2,2-dichloropropionate likewise decompose 2-chloropropionate and dichloroacetate and that strains degrading the monochloropropionate were likewise active upon the dichloroacetate. This is true of both the cultures described herein as well as the strains isolated by Jensen (4, 5), and it may be indicative of a generalized influence of chemical structure upon microbial activity. Evidence for an effect of molecular configuration upon microbial breakdown of phenoxyalkylcarboxylic acid herbicides also has been presented (1). The lack of equivalence between halogens makes it unlikely that the responsible enzyme is a non-specific dehalogenase which removes any halogen from utilizable organic nutrients.

The utilization of dichloropropionate without halide liberation is rather surprising. The likeliest explanations for the phenomenon are that the organically bound Cl is never cleaved but remains in solution while a portion of the carbon chain serves as a carbon source or, alternatively, that the chlorine is released in a volatile organic or inorganic form. It is also remarkable that microorganisms not only tolerate approximately 0.05 *M* iodoacetate, an inhibitor for a number of critical enzymes in cellular metabolism, but also actively degrade it. Further studies should reveal the pathway of the decomposition and the means by which these microorganisms develop in the presence of metabolic poisons.

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A MINIMAL SYNTHETIC MEDIUM SUPPORTING GROWTH OF A MICROCOCCUS SPECIES[1]

I. J. McDonald

Abstract

Nutritional requirements of a *Micrococcus* species (*M. freudenreichii*, A.T.C.C. No. 407) were studied. The organism required glutamic acid, thiamine, biotin, magnesium, iron, and potassium for growth. Cells from such a synthetic medium were shown to contain methionine indicating that inorganic sulphur was used. Glutamic acid could not be replaced with glutamine (unheated), aspartic acid, asparagine, nor ammonium salts. The relationship of nutritional requirements of micrococci and staphylococci to classification is discussed.

Introduction

During the course of investigations (7) on proteinase production by *Micrococcus freudenreichii*, A.T.C.C. No. 407, it became advantageous to grow the organism in a synthetic medium. Although many reports were found on the nutritional requirements of *Micrococcus lysodeikticus* (20, 21) and *Staphylococcus aureus* (5, 6, 8, 11, 14, 18, 19), only one paper gave nutritional requirements for a strain of *M. freudenreichii* (12). Since this latter organism was more fastidious than staphylococci and other micrococci and did not resemble strain No. 407 in its characteristics, an investigation of some nutritional requirements of strain No. 407 was undertaken. As the work progressed it became evident that strain No. 407 was not a true representative of the species *M. freudenreichii* but that it did resemble closely other members of the staphylococcus–micrococcus group. This paper records some nutritional requirements of this strain and gives the composition of a minimal synthetic medium that supported good growth.

Methods

Cultures of *Micrococcus freudenreichii* A.T.C.C. No. 407 (hereafter referred to as *Micrococcus* sp.) were maintained by monthly transfer on slants of blood agar base (Difco). The organism was characterized using procedures in the *Manual of Microbiological Methods* (15), i.e., carbohydrate fermentations, hemolytic activity (sheep red cells), nitrite from nitrates, and the method of Smith *et al.* (16), for the production of urease. In addition, casein hydrolysis was detected in blood agar base plus skim milk; catalase was detected by gas evolution upon the addition of hydrogen peroxide (superoxol, Merck) to slant cultures. Coagulase was tested for by adding a heavy suspension in saline of cells from a slant culture to citrated rabbit plasma diluted 1:4 and incubating at 37° C for 4 hours. Ammonia production by cultures in peptone and glutamic acid media was detected by Nessler's reagent. Ability of the organism to grow in media containing salt was determined using tryptone yeast extract broth (7).

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Inocula for assays were grown in tryptone yeast extract broth for 18 to 20 hours. Cells were collected, washed three times with, and suspended in, distilled water (or deionized water when mineral requirements were to be determined), and a 0.2% inoculum was used.

The complete medium used for initial experiments on the nutritional requirements of the organism had the following composition: DL-alanine, L-arginine, DL-aspartic acid, L-cystine, glycine, L-histidine, DL-isoleucine, L-leucine, L-lysine, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine, and DL-valine each 0.5 g; L-glutamic acid 1.0 g; magnesium sulphate 200 mg; potassium dihydrogen phosphate 500 mg; tris(hydroxymethyl)aminomethane (TRIS) 500 mg; sodium acetate 100 mg; adenine sulphate, guanine hydrochloride, xanthine, uracil each 20 mg; nicotinic acid, riboflavin, calcium pantothenate, pyridoxine hydrochloride each 0.8 mg; thiamine hydrochloride, pyridoxamine dihydrochloride, pyridoxal hydrochloride, *p*-aminobenzoic acid each 0.4 mg; folic acid 20 μg; and biotin 4.0 μg per liter adjusted to pH 7.0–7.2.

Nutritional requirements were determined in glassware cleaned with chromic acid and washed with distilled or deionized water. Mineral requirements were determined using media prepared with deionized distilled water. Media were dispensed in aliquots of 25 ml into 125-ml Erlenmeyer flasks and were sterilized at 121° C for 15 minutes. Aqueous solutions of maltose (10%) were sterilized separately and glutamine solutions were sterilized by passage through a Seitz filter. After inoculation, flasks were shaken at room temperature (25° C) on a platform shaker operating at about 100 r.p.m. Growth was determined by measuring the optical density of cultures at 660 mμ using a Coleman Junior spectrophotometer. Blanks consisted of supernatants of cultures clarified by centrifugation.

Methionine was assayed microbiologically (1) using *Leuconostoc mesenteroides* (A.T.C.C. No. 8042) as the test organism and dehydrated methionine assay medium (Difco) as prescribed by Steele *et al.* (17).

Results

Microscopic examination of the organism, grown 24 hours on blood agar base, showed Gram-positive, nonsporeforming, nonmotile cocci occurring in tetrads and irregular clusters. The organism was aerobic, growing better in shaken than in static cultures. It produced catalase and proteinase but not urease, coagulase, or hemolysin. Nitrate was not reduced to nitrite; ammonia was produced from peptone and glutamate. Cultivation of the organism in tryptone yeast extract broth containing bromcresol purple indicator resulted in an alkaline reaction with the following: arabinose, ribose, xylose, rhamnose, galactose, glucose, fructose, cellobiose, maltose, melibiose, lactose, sucrose, trehalose, raffinose, glycerol, mannitol, starch, and inulin. The organism grew in media containing 0 to 6% but not 8% sodium chloride.

The above characteristics indicated that the organism is a member of the genus *Micrococcus* as defined in *Bergey's Manual of Determinative Bacteriology* (2) but that it does not fit the description of *M. freudenreichii* or any of the

other species listed. Shaw *et al.* (13) placed *M. freudenreichii* A.T.C.C. No. 407 in group 5 of their classification and suggested that this group of organisms be called *Staphylococcus afermentans*. However, in view of the uncertain classification of this strain, it has been designated as *Micrococcus* sp. in the present work.

Preliminary results indicated that the micrococcus would grow in the complete medium but would not grow if the vitamins or amino acids were left out. Adenine, guanine, xanthine, and uracil were not required for growth and the effect of individual vitamins was determined in a medium without purines and pyrimidines (medium A, Table I). Of the 10 vitamins tested, three affected growth of the organism. Thiamine was required for growth; biotin or folic acid singly or together did not replace thiamine. In the presence of thiamine, biotin was stimulatory whereas folic acid was somewhat inhibitory. In other experiments, attempts to replace thiamine with any other single B-vitamin were not successful. It appears, therefore, that in an amino acid medium, the only single vitamin required for growth was thiamine. It must be noted that serial cultivation was not attempted in any of the above experiments. Results represent the ability of washed cells from a non-synthetic medium to grow under the conditions described.

TABLE I

Effect of vitamins on growth of *Micrococcus* sp. in amino acid medium (64 hours at 25° C)

Addition to basal medium*	Growth, O.D. at 660 mμ
Thiamine (0.2 μg/ml)	0.23
Biotin (0.004 μg/ml)	0.05
Folic acid (0.01 μg/ml)	0.04
Thiamine + biotin	0.33
Thiamine + folic acid	0.16
Biotin + folic acid	0.05
Thiamine + biotin + folic acid	0.28

NOTE: All data in this and subsequent tables is representative of at least three trials.

*Basal medium A: $MgSO_4$ 20 mg, $NaC_2H_3O_2.3H_2O$ 10 mg, KH_2PO_4 50 mg, TRIS 50 mg, per 100 ml, and 18 amino acids (see text for concentrations).

The amino acid requirements for growth of the organism were determined using medium B (Table II) containing inorganic salts, sodium acetate, TRIS, thiamine, biotin, and 18 amino acids. Growth of the organism was drastically reduced by deleting glutamic acid, glycine, or methionine. Omission of other amino acids had little effect on growth although results were somewhat variable. In other experiments, when only these three "essential" amino acids were present in the medium, growth was about one-fifth normal. In the above experiments, as in those with vitamins, attempts were not made to serially subculture the organism.

The minimum nitrogen source for growth of the organism was studied using medium B with sodium chloride in place of sodium acetate. Some growth, which was maintained on subcultivation, occurred when glutamic acid was the sole source of nitrogen and carbon. Addition of glucose to this medium was

stimulatory; addition of ammonium phosphate was inhibitory. In a medium containing glucose, thiamine, biotin, and salts ($MgSO_4$, KH_2PO_4, and NaCl), glutamic acid supported growth; ammonium phosphate, unheated glutamine, aspartic acid, or asparagine did not. In such a glucose – glutamic acid medium neither biotin (0.01 μg/ml) nor thiamine (0.5 μg/ml) alone supported growth but when both were present, the organism grew and could be transferred through at least three subcultures. Under these conditions, biotin could be replaced by aspartic acid plus Tween 80 as a source of oleic acid (cf. ref. 20). In other experiments, growth was maintained for three serial transfers in the glucose – glutamic acid medium with thiamine at 0.005 μg/ml and biotin at 10 $\mu\mu$g/ml.

TABLE II

Effect of amino acids on growth of *Micrococcus* sp. (40 hours at 25° C)

Experiment A		Experiment B	
Amino acid omitted*	Growth, O.D. at 660 mμ	Amino acid omitted*	Growth, O.D. at 660 mμ
None	0.48	None	0.44
Arginine	0.46	Alanine	0.40
Aspartic acid	0.41	Cystine	0.39
Histidine	0.44	Glutamic acid	*0.12*
Isoleucine	0.42	Glycine	*0.13*
Leucine	0.47	Phenylalanine	0.41
Lysine	0.35	Proline	0.43
Methionine	*0.17*	Threonine	0.44
Serine	0.51	Tryptophan	0.44
Valine	0.57	Tyrosine	0.43

*Basal medium B: $MgSO_4$ 20 mg, $NaC_2H_3O_2.3H_2O$ 10 mg, KH_2PO_4 50 mg, TRIS 50 mg, thiamine HCl 0.08 mg, biotin 0.8 mg, per 100 ml, and 18 amino acids (see text for concentrations).

Growth of *Micrococcus* sp. in synthetic medium did not appear to be due to a selection of mutants as indicated by the fact that cells grown in tryptone yeast extract broth and washed twice with water gave counts of 1.6×10^9 on tryptone yeast extract agar and 1.9×10^9 on glucose – glutamic acid agar. Previously, Wolin and Naylor (21) showed that growth of *M. lysodeikticus* in synthetic medium involved a selection of one cell in about 400,000.

Mineral requirements for growth were studied using medium C (Table III) containing glutamic acid, biotin, thiamine, sodium chloride, potassium phosphate, and maltose. (The organism grew slightly better with maltose than

TABLE III

Effect of magnesium on growth of *Micrococcus* sp. (40 hours at 25° C)

Added magnesium* (μg/ml)	Growth, O.D. at 660 mμ
0.0	0.07
0.02	0.12
0.04	0.17
0.1	0.35
0.2	0.52

*Basal medium C: glutamic acid 500 mg, thiamine HCl 200 μg, biotin 4.0 μg, NaCl 0.5 g, KH_2PO_4 0.2 g, $FeSO_4.7H_2O$ 0.1 mg/100 ml. Maltose 500 mg/100 ml added after separate sterilization.

with glucose.) Preliminary experiments, using citrate and 2,2′-bipyridine as chelating agents, indicated that magnesium and iron were required for growth. In media prepared with deionized distilled water, magnesium was found to be essential and at least 0.2 μg Mg^{++}/ml was required for maximal growth (Table III). In similar experiments, magnesium chloride was as effective as magnesium sulphate but neither manganese nor calcium could replace magnesium. In addition to magnesium, the organism required iron and potassium for growth (Table IV). Maximal growth occurred with about 1.0 microgram of Fe^{++} per milliliter and about 6.0 micrograms of K^+ per milliliter under the conditions used. No attempt was made to determine if sodium, chloride, phosphate, or sulphate ions were required for growth. However, methionine was detected in acid hydrolyzates of cells grown in synthetic medium indicating that sulphate ion was used by the organism.

TABLE IV

Effect of iron and potassium on growth of *Micrococcus* sp. (40 hours at 25° C)

Added iron* (μg Fe^{++}/ml as $FeSO_4$)	Growth, O.D. at 660 mμ	Added potassium† (μg K^+/ml as KCl)	Growth, O.D. at 660 mμ
0.0	0.05	0.13	0.06
0.02	0.12	0.65	0.10
0.2	0.34	1.3	0.12
1.0	0.37	6.5	0.32
2.0	0.34	13.0	0.35

*Basal medium C (Table III): with $MgSO_4$ 20 mg/100 ml but without $FeSO_4.7H_2O$.
†Basal medium C (Table III): with $MgSO_4$ 20 mg/100 ml and with KH_2PO_4 replaced by NaH_2PO_4 0.2 g/100 ml.

Discussion

The nutritional requirements of *Micrococcus* sp. are quite simple, glutamic acid serving as a sole source of carbon and nitrogen in the presence of biotin, thiamine, and salts. This organism, *Micrococcus lysodeikticus* (21), and *Micrococcus sodonensis* (10) have similar nutritional requirements and they are apparently related to staphylococci (3, 5, 6, 8, 14, 19). *Micrococcus* sp. required potassium for growth and, therefore, can be added to the list of bacteria known to require this element (9). The organism required iron, an element that was required by *M. lysodeikticus* (21) also, but one which does not seem to have been studied for staphylococci. *Micrococcus* sp. did not appear to require calcium or manganese and its magnesium requirement was not satisfied by these elements.

The present classification of the genus *Micrococcus* depends partly on the ability of the organisms to ". . . satisfy their needs for nitrogen and carbon from such simple compounds as ammonium phosphate and glucose, ammonium tartrate or asparagine and glucose" (2). *Micrococcus* sp. was able to utilize glutamic acid but not glutamine, aspartic acid, asparagine, or ammonium phosphate as a nitrogen source. This suggests that the organism, and possibly other micrococci and staphylococci, have rather specific nitrogen requirements and that chemical complexity of a compound is not necessarily a valid criterion of its availability to an organism. Other workers (4, 13) have suggested that

growth of micrococci with ammonium salts may depend upon carry-over of nutrients in the inoculum. The extremely small amounts of biotin and thiamine required by *Micrococcus* sp. would seem to emphasize that caution must be exercised to eliminate such carry-over in evaluating growth in minimal media. The vitamin requirements of the organism seemed to be related to the composition of the medium (e.g., biotin replaced by oleic acid plus aspartic acid) and this also may be significant in the classification of this organism and of other micrococci. It is concluded, therefore, that further critical investigations of nutritional requirements of micrococci and staphylococci are needed to clarify their classification.

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THE EFFECT OF pH ON THE DEVELOPMENT OF BACTERIA IN WATER STORED IN GLASS CONTAINERS[1]

LOUISE F. POTTER

Abstract

Storing water samples in buffers results in increases of the total numbers of organisms. The pH and the length of the incubation period affect not only the size of population but also the kinds of organisms in the population.

Introduction

Bacterial populations increase in stored water (1, 10, 11, 15) although the number of kinds of bacteria present may decrease. The chemicals, both organic and inorganic, present in the water are determinants of the increase in the populations. ZoBell and Anderson (15) indicate that the ratio of volume to surface was important in this increase and some of Whipple's results (12) support this. Taylor (10) indicates that temperature has significant effect on multiplication as does agitation of samples. Buffers according to Garvie (4) and inorganic chemicals according to Gunter (5) and Noble and Gullans (6) affect the viability and survival of pure cultures of bacteria, such as *Escherichia coli*.

The effect of pH on the growth and metabolism of pure cultures is well known. Winslow and Falk (14) and Winslow and Brooke (13) examined the effect of pH on the survival of pure cultures in water and dilute solutions. The effect of pH on mixed populations as they occur in natural, untreated water is not well annotated. The following experiments, therefore, were undertaken to resolve this point.

Materials and Methods

Potable spring water, available as unchlorinated tap water, was used for the first experiment (Table I). Erlenmeyer flasks of 500-ml capacity were cleaned by boiling and washing them in detergent, then rinsing them in tap water and again with distilled water. They were plugged with cotton and sterilized in a hot-air oven. Tap water in 250-ml quantities was added to each sterile flask and the pH adjusted by the addition of 1 *N* NaOH or sterile 10% H_3PO_4. These cultures were incubated at room temperature for a total incubation period of 19 days.

Although the pH of the stored water was adjusted during the incubation period, the desired pH was not maintained. Consequently buffers were used in succeeding experiments (Tables II, III, IV). Fifty milliliters of sterile buffer in a 250-ml Erlenmeyer flask was inoculated with 50 ml of water. For this series of experiments water from a small stream was used as the inoculum. This stream water, unlike the spring water of the first experiment, was not potable. The number of bacteria per milliliter was much higher in the stream water than in the spring water (Tables III, IV).

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TABLE I

Organisms in tap water inoculum stored at adjusted pH levels

Initial pH	Final pH	Total number of bacteria per cc × 10^3	Per cent yellow-orange chromogens
	Inoculum		
	8.2	0.003	66
	After 3 days' incubation		
8.2	8.2	31	0
5.0	5.0	141	0
5.5	6.8	2,880	7
6.0	6.8	469	0.4
6.5	7.6	1,360	2
7.0	8.3	2,980	3
7.5	8.2	657	1
8.0	8.1	680	0
8.5	8.2	—	—
9.0	8.2	700	0
	After 19 days' incubation		
8.2	8.2	2,020	39
5.0	4.0	380	0
5.5	4.0	—	—
6.0	6.9	1,190	25
6.5	8.2	1,345	3
7.0	8.0	2,570	37
7.5	8.0	565	18
8.0	8.0	5,100	58
8.5	8.3	205	17
9.0	8.3	1,120	22
9.5	8.4	—	—

For pH 2, 3, 4, 5, and 6 the buffers were 0.1 *M* citric acid and 0.2 *M* disodium phosphate. In the second experiment (Table II) the buffer for pH 7 was prepared with 0.1 *N* NaOH and 0.1 *M* KH_2PO_4. For pH 8, 9, and 10 the buffers were prepared from 0.1 *M* NaOH and 0.1 *M* H_3BO_3 (2). Different buffers were used for pH 9 and 10 in the two final experiments. For pH 9 a 0.05 *M* $Na_2B_4O_7$ was used; for pH 10 a buffer of 0.05 *M* Na_2CO_3 and 0.05 *M* $Na_2B_4O_7$ was used (2).

TABLE II

Organisms in stream water inoculum buffered from pH 1.95 to 10.0

Initial pH	Final pH	Total number bacteria per cc × 10^3	Per cent pink	Per cent yellow
		Inoculum		
	—	6.8	0	20
		After 8 days' incubation		
1.95	2.19	154	36	—
2.81	3.15	466	53	—
3.90	4.15	240	17	3
4.91	5.18	3,040	—	14
6.09	6.23	2,370	—	36
6.90	7.02	2,320	—	40
8.10	7.73	167	—	28
9.05	8.35	352	—	29
10.00	8.35	171	—	—

After the incubation periods at room temperature the samples were mixed thoroughly by shaking. Three dilutions in replicates of five were plated with sodium caseinate agar (3). The plates were incubated 15 days at room temperature.

From the morphological studies the colonies were selected from the dilution counted. A spot was selected at random on a plate. Rotating clockwise, every colony was smeared until a minimum of 60 or a maximum of 100 smears was obtained. The slides were stained by Hucker's modification of the Gram stain.

Results and Discussion

It can be seen in Table I that there was bacterial multiplication in the stored spring water regardless of pH. The adjustment of the acidity by addition of NaOH or H_3PO_4 was not satisfactory. Within 3 days the samples with a pH below 8.0 showed an increase in alkalinity and those adjusted above 8.0, an increase in acidity. The samples were readjusted after 3 and 6 days of incubation but the pH was not maintained. The adjustment of the pH resulted in the stored samples being thoroughly mixed twice during the incubation period. This agitation may have increased multiplication (10, 12). Even so, in 3 days of quiescent incubation the number of bacteria per milliliter increased a minimum of 10 thousand to almost 1 million times. Aside from the sample adjusted to pH 8.5, the control sample showed the smallest increase in population after 3 days' incubation. After 19 days there were further changes in the size of the populations, usually being very large in comparison with that of the inoculum.

Because the initial number of bacteria per milliliter of the control was low, the percentage of chromogens present was not considered significant. Generally the percentage of chromogens was still low after 3 days' incubation, but it increased significantly by 19 days. This indicates that the total number of organisms per milliliter is not the single measure of change in population. Variation in rate of multiplication of different organisms is well known. This may be significant in explaining the apparent change in the character of populations (Table I).

The first experiment demonstrated the multiplication of bacteria in stored waters, even though the adjusted pH was not maintained. It further indicated that the pH was an important determinant of the size and nature of the population. It was obvious that buffers were necessary if a given pH was to be maintained. In corroboration of this, flasks containing 50 ml of stream water and 50 ml of buffer were incubated for 8 days (Table II). The pH of each sample was measured with a Beckman pH meter at the beginning and conclusion of the incubation period. The buffers were adequate for maintaining the desired pH within a sufficiently limited range, except for pH 9 and 10. For these levels the work was repeated using sodium borate for pH 9 and a mixture of sodium borate and sodium carbonate for pH 10. The pH was checked every 2 days. It was necessary to add sodium borate to the pH 9 and sodium carbonate to the pH 10 during incubation, yet these buffers proved the most satisfactory of many tested.

The increase in population was large for these samples (Table II). The greatest increase occurred at pH 5. The highest percentage of bacterial chromogens was found at pH 7. At pH 2 and 3 the population was composed primarily of yeasts.

The stream water used as inoculum for the third experiment (Table III) had a smaller population of different composition from that of the second experiment. Samples were plated after 8 and 15 days' incubation. At pH 2, 3, and 10 the population per milliliter decreased. The largest population of all samples was found after 8 days in water buffered at pH 6. After 15 days the greatest population was found at pH 5. Not many samples contained chromogens but those that did had significantly high percentages after 8 days. At the end of the incubation period there were additional samples showing chromogens.

TABLE III

Organisms in stream water inoculum with small population, stored in buffers from pH 2.10 to 9.99

Initial pH	Final pH	Total number of bacteria per cc $\times 10^3$	Per cent pink	Per cent yellow
		Inoculum		
	7.58	0.54	11	7*
		After 8 days' incubation		
2.10	2.50	—	—	—
3.02	3.30	0.28	71.5	0
4.06	4.30	2.64	0	0
4.98	5.38	133	0	0
6.00	6.33	1,090	0	0
7.05	7.18	70	0	35
7.98	8.00	2.95	0	0
8.96	8.95	0.9	0	27
9.99	9.65	—	0	—
		After 15 days' incubation		
2.10	2.29	—	—	—
3.02	3.18	—	—	—
4.06	4.30	22	14	21
4.98	5.40	313	0	10
6.00	6.38	252	0	19
7.05	7.29	197	0	64
7.98	8.12	23.5	0	0
8.96	8.98	0.76	0	0
9.99	9.63	—	—	—

*4% orange.

The fourth experiment was a repetition of the third using an inoculum containing a much larger population than all previous experiments (Table IV). After 8 days the number of bacteria per milliliter had increased except at pH 9. In many samples this increase was much smaller than the increase obtained in the preceding experiments based on inocula with fewer organisms initially. The greatest increase at 8 days occurred at pH 5. By 15 days' incubation all the samples except that of pH 7 showed a population decrease. The percentage of chromogens in the samples usually increased during the total incubation period.

TABLE IV

Organisms in stream water with large population, stored in buffers from pH 2.15 to 10.12

Initial pH	Final pH	Total number of bacteria per cc × 10^3	Per cent pink	Per cent yellow
		Inoculum		
	7.5	40.2	8	9
		After 8 days' incubation		
2.15	2.38	5,640	84	0
2.92	3.02	4,720	82	0
4.05	4.12	3,224	2	3
5.01	5.10	8,140	0.2	0
5.91	6.02	5,868	0	11
7.09	7.13	5,156	0	8
8.12	8.02	2,064	0	2
9.12	8.92	—	—	—
10.12	9.73	253	0	3
		After 15 days' incubation		
2.15	2.19	824	78	0
2.92	2.99	—	—	—
4.05	4.22	564	10	2
5.01	5.30	7,720	0	3
5.91	6.11	4,700	4	12
7.09	6.99	35,120	0	10
8.12	7.73	1,316	0	4
9.12	8.88	—	—	—
10.12	9.70	—	—	—

TABLE V

Percentages of kinds of organisms present in stored water samples with adjusted pH levels

pH	Yeasts	Rods	Cocci	Gram-positives	Gram-negatives	Gram-variables
			Sample inoculum			
	0	70	30	70	27	3
			After 8 days' incubation			
2	100	0	0	0	0	0
3	100	0	0	0	0	0
4	13	80	7	50	32	5
5	0	70	30	66	21	13
6	0	80	20	78	14	8
7	0	92	8	77	13	10
8	1	96	3	87	12	0
9	—	—	—	—	—	—
10	0	87	13	90	7	3
			After 15 days' incubation			
2	100	0	0	0	0	0
3	—	—	—	—	—	—
4	46	49	5	18	34	2
5	0	82	18	65	20	15
6	0	72	28	77	11	12
7	0	74	26	81	13	6
8	0	85	15	45	50	5
9	—	—	—	—	—	—
10	—	—	—	—	—	—

Smears of at least 60 colonies per sample were examined microscopically. Results are recorded in Table V. All colonies obtained on plates from cultures buffered at pH 2 and 3 were yeasts. Inspection of Tables IV and V shows that the yeasts represented the pink colonies and were not present in smears from the control plates. It is well known that acid media are useful in isolating yeasts. Here storing water in acid buffers allowed for a tremendous increase of yeasts per milliliter. Yeasts, present after 8 days at pH 4, increased by 15 days. In all samples at 8 days, rods were predominant over cocci. Usually the percentage of rods increased over those present in the inoculum. The pH 4 and 5 samples showed a decrease in Gram-positive organisms and all other samples, an increase. In samples from pH 5, 6, and 7 the percentage of Gram-variables increased. After 15 days the percentage of rods decreased usually and the percentage of cocci increased. The percentage of Gram-positives decreased at all pH levels except 7. At all pH levels buffered below 7, except pH 4, the percentage of Gram-negatives decreased. Gram-negative rods increased significantly at pH 8. The Gram reactions of fresh water isolates from lakes in England have been reported by Taylor (9) and for Lake Mendota by Snow and Fred (8). Gram-negative organisms are usually predominant, as noted in water samples from Flathead Lake, Montana (7). Variations do exist in different bodies of water. In the experiments reported here, the heavily contaminated stream water showed an unexpectedly high number of Gram-positive organisms which appeared to be associated with the contamination (9).

The results in Table IV indicated also that the composition of the population in a stored water sample changes during the incubation period. As a result of these experiments it is apparent that there are significant and often large increases in the number of organisms per milliliter in stored water. These increases are inversely proportional to the number of organisms per milliliter of inoculum. It is probable that no two inocula were comparable. In spite of that it appears that the pH of the sample is important in determining the size of the population within a definite incubation period. Because it was impossible to maintain the desired acidity without the use of buffers, it is impossible to evaluate the significance of the buffer components as nutrient sources.

Sometimes an increase in population is followed by a decrease. Early in the incubation period a pH below 4 or above 9 appears to be toxic and few, if any, organisms are found. The percentage of chromogens increases with increased length of incubation period at almost every pH level. This may indicate either that chromogens multiply more slowly or that they are more resistant organisms. Changes among morphological types of bacteria indicate further that the character of the population is continually changing.

The organisms were not identified so there is no specific evidence to show that the number of kinds of organisms decreases (10), even though the total number of organisms increases. If the organism is not present in sufficiently large numbers it would be difficult to determine its presence without using

enrichment methods. This is demonstrated for yeasts (Table V) which were not detected in the inoculum but became predominant in cultures maintained below pH 4.

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THE PRODUCTION OF CELLULASE BY FUNGI ON MIXED CELLULOSIC SUBSTRATES[1]

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Abstract

Using jute, alkali-treated jute, holocellulose from jute, and filter paper as substrates, a total of 124 fungi were tested for production of cellulolytic enzyme capable of hydrolyzing powdered filter paper and swollen cellulose. Of these, only 15 organisms failed to produce the enzyme on any substrate, and 48 produced cellulase on filter paper, so that in most species cellulase production could be induced only by mixed cellulosic substrates. Among such species were many that are ordinarily regarded as non-cellulolytic, while on the other hand comparatively little enzyme could be detected with several well-known cellulose decomposers strongly degrading cellulose in vivo. Factors stimulating enzyme production on mixed cellulosic substrates were micronutrients and hemicellulose. For most species, excluding a few of the strong cellulose decomposers, a partial loosening of the hemicellulose seemed to favor enzyme production best. Some cellulase was produced on free hemicellulose alone, but not on starch and sugars, although in presence of cellulose these substances stimulated enzyme production by a few ordinarily weak species; on strong cellulose decomposers the effect of these additional carbon sources was opposite, possibly due to a sparing action on cellulose.

Introduction

No comprehensive study of the fungal decomposition of cellulose was carried out until comparatively recently. In 1948, White and co-workers (27) systematically measured cellulose decomposition and loss in tensile strength of cotton fabric caused by a large number of fungal species and strains, isolated at various parts of the world, particularly during the 1939–45 war. They found activity in 18 out of 45 genera tested and showed the importance of *Aspergillus* species, previously regarded as surface organisms growing on the impurities and finishing materials. This was followed by a similar study by Marsh and co-workers (16), who examined the cellulolytic activity of more than 400 fungal isolates and showed that the ability to cause loss in tensile strength of cotton duck was widely distributed, but not universal, among the fungi in general and among the 140 isolates of *Penicillium* species in particular. In our previous work in this field (5) attention was confined to the degradation of cellulose by living fungi in the particular context of decomposition of lignocellulosic materials such as jute. Using a number of commonly occurring species it was shown that while lignin exerted an inhibitory effect, hemicelluloses and micronutrients present in jute favored decomposition, as determined by loss in weight. Jute treated with dilute alkali became much more susceptible to attack (6).

Reese, Siu, and Levinson (19) were the first to determine the cellulolytic activities of the culture filtrates of such fungi; these were grown on media

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containing carboxymethyl cellulose (CMC) and other cellulose derivatives, ground cotton duck and bleached cotton sheeting, and CMC was taken as the substrate for measuring enzyme activity. From the reaction of fungi and their enzymes towards various cellulosic substrates, they postulated two steps in the conversion of native cellulose to soluble products; according to them, the enzyme C_1, present only in truly cellulolytic fungi, converted native cellulose into linear cellulose chain molecules through the breakdown of cross-linkages, whereupon the latter were hydrolyzed into sugars by another enzyme Cx, present even in some non-cellulolytic fungi. Later, Reese and Levinson (18) experimented with a large number of microorganisms, mostly fungi, using cotton sliver, wood cellulose, alkali cellulose, hydroxyethyl cellulose, dextrin, and glucose as substrates for growth, estimating only Cx in the culture filtrates, on the basis of hydrolysis of CMC. High Cx activity was found to be generally associated with high loss of tensile strength of cotton duck, and vice versa, although there were exceptions; for example, *Myrothecium verrucaria*, causing complete loss of tensile strength, showed only a low Cx concentration in the filtrate, while *Penicillium pusillum* producing 20 times more enzyme caused only 30% strength loss. Some fungi which failed to attack cotton duck grew well on wood cellulose (which contains pentosans) and produced enzyme. This is related to our previous work (5) in which it was shown that the hemicellulose of jute had a favorable effect on the decomposition of the true cellulose. Reese and Levinson (18) also showed that Cx could be produced in the absence of cellulose or its derivatives in the medium, a finding which was confirmed by Jermyn (13) in Australia.

The present work reports the results of study of cellulolytic enzyme production undertaken as a continuation of our previous work on decomposition of cellulose and jute by living fungi. A large number of species, comprising apparently non-cellulolytic types, were included, and the effect of substances closely associated with cellulose in nature was studied.

Materials and Methods

Substrates

For enzyme production, the following substrates were used: filter paper, Whatman No. 1; jute fiber, a tossa variety from Karimpur; holocellulose (11), α-cellulose (21) and hemicellulose (22) prepared from jute by the methods of Sarkar and co-workers; and alkali jute, prepared by boiling deoiled jute in a 1% caustic soda solution, unless otherwise mentioned; the treated fiber was freed from alkali with distilled water and dilute acetic acid, followed by repeated washing till a constant pH was obtained.

For the assay of enzyme activity, two substrates were used: powdered filter paper (PF) and swollen cellulose (SC). The former was obtained by pulverizing Whatman No. 1 filter paper in a Wiley mill, using a 200 mesh sieve; the latter was prepared by swelling a sample of purified Indian cotton linters with phosphoric acid, as described by Walseth (25).

Fungi

The total number of organisms tested was 124; most were isolations from deteriorated jute and other lignified materials, and were preserved alternately on potato-dextrose agar and malt-extract agar slants. Numbers were assigned to them generally in the order of isolations, and these are given after the species name in the tables.

Enzyme Solution

For growing the fungi, 15 ml Czapek-Dox salt solution (pH 7.0) were taken in 250-ml conical flasks, some glass beads poured in to cover the bottom, and the substrate spread evenly on them; jute and alkali jute were taken in approximately 0.6-g quantities, holocellulose in 0.4-g quantities, and filter paper (7-cm circle) in approximately 0.35-g quantities. The flasks, after sterilization, were inoculated with spores of the fungi and incubated at 30° C for different periods ending roughly with the state of most active growth, namely, holocellulose 10 days, filter paper and alkali jute 13–14 days, and jute 21 days. Data on other substrates regarding quantity of substrate and duration of incubation are given along with the experiments concerned. The contents of the flasks were then filtered through sintered glass and the volume made up to 15 ml with wash water from the residue. This was used as the enzyme solution.

Enzyme Assay

Enzyme hydrolysis was carried out in duplicate 125-ml Pyrex conical flasks, containing: PF 200 mg or SC 100 mg, *N*/10 acetate buffer of pH 5.0 saturated with the antiseptic pentachlorophenol 10 ml, enzyme solution 1 ml, distilled water 9 ml. The flasks were incubated at 40° C for 18 hours in the stationary state. At the end of this period 5 ml of hydrolyzate were withdrawn and reducing sugar determined according to the micromethod of Somogyi (23). The titers were corrected by subtracting the mean titers for substrate without enzyme and enzyme without substrate. The mean titers were taken as the measure of enzyme activity and the actual amounts of sugar formed were not calculated.

Fungal growth on various substrates was sometimes indicated by figures, ranging from 0 (no growth), tr. (trace), to 4 (heavy growth).

Results

To begin with, all the fungi were tested for enzyme production on filter paper (F), holocellulose (H), jute (J), and alkali jute (AJ). The results are given in Tables I–III, which group together the organisms on the basis of failure to produce cellulolytic enzyme on one or more of the various substrates. Data from our previous experiments on loss in weight of filter paper and loss in strength of jute cloth caused by the living fungi are given in the last two columns of these tables for the sake of comparison (1, 4, 5, 9).

Out of a total of 124 fungi (89 species, 28 genera) tested, 48 fungi (36 species, 14 genera) fell in the first series, comprising organisms producing some enzyme on filter paper (α-cellulose), which means that 39% of the

TABLE I

Organisms producing enzyme on filter paper

Serial No.	Species and ref. No.	Enzyme titer								Loss in wt. of filter paper, %	Loss in tensile strength of jute cloth, %
		Filter paper		Holocellulose		Jute fiber		Alkali jute			
		PF	SC	PF	SC	PF	SC	PF	SC		
1	*Penicillium variabile* 121	3.30	6.30	3.10	5.60	4.60	7.85	4.60	8.90	32.2 (9)	
2	*Aspergillus terreus* 6.2	3.30	7.80	2.85	5.90	3.80	7.00	6.50	9.20	80.4	
3	*Penicillium janthinellum* series 153	3.30	11.25	2.15	5.30	3.40	5.80	3.20	6.30		
4	*Aspergillus ustus* 29	3.30	8.10	3.05	5.60	2.50	5.80	1.00	2.30	30.0 (1)	
5	*Penicillium vermiculatum* 77.32	3.10	5.40	1.15	1.95	1.30	3.20	2.90	6.90	6.5 (9)	24.0 (4)
6	*Penicillium variabile* 121.1	3.05	12.25	1.50	2.90	2.25	4.70	0.70	3.90	38.0 (9)	
7	*Penicillium janthinellum* series 153.1	2.70	6.05	2.30	4.90	4.55	7.45	2.80	5.20	51.2 (9)	
8	*Penicillium* sp. 98	2.55	8.25	1.70	3.40	5.20	8.10	4.10	6.50	50.0 (9)	
9	*Aspergillus fumigatus* 4	2.05	8.50	1.75	3.20	3.80	6.20	2.40	4.30	70.2 (5)	32.0 (4)
10	*Aspergillus terreus* 6.1	2.00	6.60	1.90	2.40	2.40	3.70	5.20	7.30		
17	*Chaetomium globosum* 79	0.45	2.55	0.60	1.45	0.90	4.05	1.10	3.60	46.0 (1)	37.0 (4)
19	*Myrothecium verrucaria* 139	0.40	1.10	1.40	2.00	0.60	2.60	0.20	0.75		68.0 (4)
21	*Penicillium citrinum* 37	0.35	1.50	4.20	6.15	0.45	1.45	1.90	4.70	9.0 (5)	20.0 (4)
22	*Trichoderma viride* 24	0.30	0.40	0.50	1.15	1.40	2.75	0.10	0.20	6.0 (5)	7.0 (4)
26	*Penicillium variabile* 121.2	0.30	1.00	2.50	4.10	1.75	3.75	4.30	8.20	22.4 (9)	
28	*Alternaria* sp. 60	0.25	0.85	0.50	1.60	0.40	2.15	0.40	1.10		51.0 (4)
30	*Penicillium oxalicum* 96	0.25	0.40	0.90	2.30	5.70	9.65	0.20	0.80	21.6 (9)	
31	*Aspergillus sydowi* 44	0.25	0.35	0.20	2.30	0.00	0.00	0.25	0.40	33.7 (5)	
33	*Trichoderma viride* 24.1	0.20	0.40	0.15	0.30	0.10	0.20	0.10	0.30		
34	*Penicillium luteum* 28 (non-ascosporic)	0.20	1.55	2.10	4.00	0.40	2.40	5.30	11.50	0.5 (9)	31.0 (4)
36	*Penicillium cyaneum* 124	0.20	1.10	2.00	4.00	4.60	7.80	0.80	2.70	0.5 (9)	
40	*Chaetomium indicum* 75	0.10	0.90	0.80	2.00	0.90	4.00	0.70	1.40	40.0 (5)	53.0 (4)
41	*Penicillium vermiculatum* 77.62	0.10	2.75	1.05	1.55	2.40	5.60	1.20	4.40	70.7 (5)	18.0 (4)
44	*Penicillium verruculosum* 158	0.10	0.20	0.80	1.40	5.60	10.60	0.10	0.50	12.9 (9)	
47	*Penicillium funiculosum* series 150	0.00	0.40	4.50	6.90	0.40	0.80	1.60	2.80	36.2 (9)	

fungal isolates produced some enzyme on pure cellulose. The 10 most potent of these organisms as well as a few others discussed in the text are arranged in Table I, in the order of the PF titer of their enzymes derived from growth on filter paper. The order of enzyme production varied from substrate to substrate and some of the fungi producing relatively little enzyme on filter paper produced large quantities on other substrates, e.g. *Penicillium citrinum* 37 and *P. funiculosum* series 150 on holocellulose; *P. oxalicum* 96, *P. cyaneum* 124, and *P. verruculosum* 158 on jute fiber; and *P. variabile* 121.2 and *P. luteum* 28 on alkali jute. Only one organism, *Chaetomium brasiliense* 117.1, produced cellulase on filter paper but none from holocellulose or jute. An analysis based either on the number of times that maximum and minimum amounts of enzymes were produced on these substrates (sum of PF and SC titers) or on the sum of the actual titers for each fungal substrate showed that their suitability decreased in the order: AJ, J, H, F, which means that alkali jute was the most favorable substrate for the maximum number of fungi (taking PF titers alone however, jute was more potent than alkali jute). Now, it has already been mentioned that jute becomes more susceptible to fungal attack on alkali treatment (6), so that the production of more enzyme on alkali jute than on untreated jute is not unexpected. It is also known however from previous studies that jute holocellulose is much more easily decomposed by living fungi than the whole fiber, and this is attributed to the removal of lignin; the production of more enzyme from jute than from holocellulose was therefore somewhat puzzling initially, but it can be satisfactorily explained on the basis of the micronutrients in jute which, as will be shown later (Table VII), have a marked oligodynamic action on enzyme production by some species; it was found that when the above analysis was carried out without four such species, total enzyme titer worked out to be higher for holocellulose than for jute.

It is apparent from Table I that although correlation can sometimes be established between enzyme production and apparent cellulolytic activity of the living fungus, striking differences may also exist. Typical examples are found in *Alternaria* sp. 60 and *Trichoderma viride* 24, loss in strength of jute fabric and cellulase production on jute being in inverse ratio between them; *Chaetomium indicum* 75 and *Penicillium vermiculatum* 77.62 gave low enzyme but high weight loss on filter paper, but the situation was reversed with strain 77.32 of the latter species. A more striking example of strain variation is seen in the three isolates of *Penicillium variabile*, which seems to deserve its name on more counts than morphological characteristics; all three strains were isolated from jute at the same time, yet not only was there great variation in the amount of enzyme produced on the various substrates, but also the PF titer of No. 121.1 decreased in the order F, J, H, AJ, while that of No. 121.2 fell exactly in the reverse order; one strain (No. 121) produced near-maximum amounts of enzyme on all four substrates (though, incidentally, in actual cellulose consumption it was inferior to such organisms as *Aspergillus terreus* 6.2 and *Penicillium janthinellum* 153.1), yet this species has

TABLE II

Organisms not producing enzyme on filter paper

Serial No.	Species and ref. No.	Enzyme titer						Loss in weight of filter paper, %	Loss in tensile strength of jute cloth, %
		Holocellulose		Jute fiber		Alkali jute			
		PF	SC	PF	SC	PF	SC		
1	*Penicillium roseo-purpureum* 148	5.50	11.70	0.50	1.20	8.50	12.10	6.0 (9)	
2	*Penicillium rubrum* 127	4.90	8.00	0.30	0.75	5.10	8.70	3.0 (5)	47.0 (4)
3	*Penicillium steckii* 137	4.50	8.20	0.20	0.60	1.50	3.80	13.3 (9)	
4	*Penicillium vermiculatum* 77.10	2.50	4.40	1.70	3.00	5.60	8.90	0.6 (9)	15.0 (4)
5	*Penicillium notatum* 138	2.40	4.50	0.70	1.30	0.90	1.90	5.7 (9)	
6	*Chaetomium funicola* 112	2.20	5.00	1.95	4.10	0.00	0.00		43.0 (4)
8	*Chaetomium dolichotrichum* 111	1.40	2.60	1.00	2.90	0.40	1.50		60.0 (4)
10	*Chaetomium funicola* 112.1	1.15	2.00	0.60	2.40	0.00	0.20		
11	*Chaetomium* sp. 115	1.00	1.80	1.30	3.30	0.20	0.70		28.0 (4)
12	*Chaetomium cochliodes* 110	0.70	1.50	0.40	1.30	0.30	0.80		38.0 (4)
14	*Stachybotrys atra* 70.1	0.60	1.20	1.00	1.70	0.00	0.00		47.0 (4)
15	*Chaetomium cancroideum* 108	0.60	1.20	0.75	2.70	0.30	1.00		35.0 (4)
16	*Stachybotrys atra* 70	0.40	1.00	2.40	6.20	0.00	0.00		
21	*Memnoniella echinata* 133	0.10	0.65	1.60	4.40	0.00	0.20		
22	*Chaetomium pachypodioides* 114	0.10	0.20	0.55	1.10	0.00	0.00		
23	*Chaetomium ochraceum* 109	0.10	0.30	0.35	1.40	0.00	1.20		31.0 (4)
24	*Chaetomium cochliodes* 110.1	0.10	0.20	0.10	0.60	0.10	0.10		
25	*Chaetomium microcephalum* 113	0.00	0.00	0.60	1.20	0.00	0.00		
27	*Memnoniella echinata* 73	0.00	0.00	0.10	0.70	0.00	0.00		35.0 (4)
28	*Memnoniella echinata* 73.1	0.00	0.00	1.50	3.40	0.00	0.00		

been found to be non-cellulolytic by Marsh *et al.* (16). Among the organisms belonging to species which had shown powerful in vivo cellulolytic activities in previous experiments by various workers (5, 12, 24), but gave rather poor enzyme concentrations in the present studies, mention should be made of *Aspergillus sydowi* 44, *Myrothecium verrucaria* 139, *Chaetomium globosum* 79, and *C. indicum* 75. Crushed mycelial extracts of these *Chaetomium* species also failed to show any enzyme activity. Strong fiber decomposition coupled with poor Cx enzyme production by *C. globosum* has also been noted by Reese and Levinson (18).

Of the 22 species in series 1 giving rather high titers (PF titer above 3 ml) when grown on one or more of the four substrates, as many as 21 belonged to *Aspergillus* and *Penicillium*. This is in keeping with their position relative to other genera as regards both cellulose decomposition and frequency of occurrence on mildew-damaged cellulosic materials (7, 15).

Other species falling in series 1 were the following, their relative positions being indicated by serial numbers in brackets: *Aspergillus nidulans* (11), *A. flavipes* (16), *A. terreus* (32), *A. carneus* (38), *A. proliferans* (48), *Cephalosporium* sp. (20), *Chaetomium brasiliense* (18), *C. indicum* (23), *C. globosum* (29), *Curvularia lunata* (39), *Fusarium* sp. (15), *Helminthosporium* sp. (42), *Heterosporium* sp. (35), *Monilia sitophila* (43), *Penicillium brefeldianum* (12, 13), *P. canescens* series (14), *P. vermiculatum* (27), *P. citrinum* (45), *Penicillium* sp. (46), *Stemphylium* spp. (24, 25), *Stysanus* sp. (37).

Series 2 comprised 29 fungi (22 species, 10 genera) which produced no enzyme only on filter paper. Only five of these (all *Penicillium* species) produced from hemicellulose or alkali jute enzyme solutions giving a PF titer of 2.4 ml or more, and these along with members of the genera *Chaetomium*, *Stachybotrys*, and *Memnoniella*, which are often associated with fiber decomposition, are arranged in Table II in order of the PF titer of the hemicellulose enzyme. Once again it is seen that strong cellulose decomposers, as judged from loss in strength of jute fabric, often produced relatively small quantities of cellulase. It is also significant in this connection that many of the *Chaetomium* species grew heavily on filter paper, though of course producing no enzyme. On the other hand, the two mutually allied fungi *Stachybotrys* and *Memnoniella* showed little or no growth on filter paper and alkali jute, though jute gave better growth. Such differences in growth (and similarly, in enzyme production and strength loss) may sometimes be due to the difference in micronutrient contents between filter paper and alkali jute on the one hand and jute on the other, and *M. echinata* 73 is definitely known to have developed biotin deficiency since its isolation (10). However, the explanation of the behavior of *Chaetomium* species and others mentioned above which showed strong evidence of attack but poor enzyme production on the same substrate is not so simple.

Series 2 was analyzed like series 1 as regards general suitability of the three substrates for enzyme production. A difference was noticed from the sequence derived from Table I in that total titer fell in the order H, AJ, J. Elimination

TABLE III

Organisms not producing enzyme on filter paper and jute

Serial No.	Species and ref. No.	Enzyme titer				Loss in weight of filter paper, %	Loss in tensile strength of jute cloth, %
		Holocellulose		Alkali jute			
		PF	SC	PF	SC		
1	*Aspergillus niger* 3	3.80	8.50	2.40	6.70		
2	*Penicillium wortmanni* 126.24	3.60	6.00	4.10	6.90	2.3 (9)	21.0 (4)
3	*Penicillium wortmanni* 126.78	3.50	7.40	4.20	8.20	2.0 (9)	
4	*Penicillium wortmanni* 130.64	2.20	4.90	1.50	3.00	0.9 (9)	43.0 (4)
5	*Penicillium wortmanni* 126.31	2.10	5.20	4.90	9.20	1.0 (5)	31.0 (4)
7	*Penicillium wortmanni* 130.30	1.90	4.65	1.40	3.70	0.6 (9)	32.0 (4)
9	*Penicillium variabile* 34	1.70	6.00	2.30	9.90	71.0 (1) 1.7 (9)	20.0 (4)
11	*Aspergillus atropurpureus* 92	1.65	5.35	9.20	13.50		
15	*Penicillium* sp. 40	1.15	2.35	0.40	0.70	55.0 (1) 0.0 (9)	24.0 (4)
25	*Helminthosporium* sp. 17	0.40	0.75	0.25	0.85	20.0 (5)	34.0 (4)
30	*Chaetomium brasiliense* 117	0.00	0.35	0.40	0.60		29.0 (4)

of species particularly sensitive to jute extract (Table VII) from the analysis narrowed the gap between the figures for jute and alkali jute, but did not reverse the sequence.

Apart from those mentioned in Table II, other species in this series were: *Acontium* sp. (27), *Alternaria* sp. (13), *Aspergillus japonicus* (7), *Cephalosporium* sp. (19), *Gliomastix convoluta* (17), *Penicillium implicatum* series (9), *P. vermiculatum* (18, 20), unidentified No. 119 (21).

In Table III are arranged as before some of the 32 organisms (25 species, 8 genera) that failed to produce any enzyme on both jute and filter paper (series 3). All except one (*Chaetomium brasiliense* 117) also showed correspondingly poor growth on those two substrates. It appears that the number of species ordinarily unable to attack jute or pure cellulose, but able to attack jute-cellulose and to elaborate cellulase under special circumstances, is quite considerable. Total titers (PF and SC) on alkali jute were on the whole somewhat greater than on holocellulose, as with pure cellulose decomposers (Table I). A very high titer on alkali jute was given by *Aspergillus atropurpureus* 92 and the highest titers on holocellulose were derived from *A. niger* 3, both of which belong to the species group *A. niger*, whose members are often seen to grow on vegetable textile materials under various conditions.

All the six strains of *Penicillium wortmanni* fell into this series and many of them gave high enzyme titers; this is to be compared with the considerable loss in fabric strength previously caused by many of them and the present poor growth on jute and cellulose. It seems that they have lost the power of attacking unmodified jute in continued subculture. Three other species in Table III deserve mention for similar degeneration, namely, *Penicillium variabile* 34, *Penicillium* sp. 40, and *Helminthosporium* sp. 17, all of which are old isolations (more than 16 years).

Other species belonging to series 3 are given in the following list, along with their relative position: *Aspergillus flavus* (19), *A. versicolor* (20), *A. oryzae* (21), *A. unguis* (28), *A. chevalieri* (31), *Botrytis cinerea* (24), *Chaetomium aureum* (26), *Cladosporium* sp. (27), *Penicillium implicatum* (6), *P. purpurogenum* (8), *Penicillium* sp. (10), *P. adametzi* (13), *P. wortmanni* (14), *P. simplicissimum* sp. (16), *P. vermiculatum* (17, 22), *P. fellutanum* (18, 23), *Penicillium* sp. (32), *Scopulariopsis* sp. (12), unidentified No. 118 (29).

Fungi producing enzyme on one substrate alone were only nine in number (eight species, six genera). *Acontium* sp. 155, *Chaetomium microcephalum* 113, and *Memnoniella echinata* 73 and 73.1, which gave cellulase on no other substrate but jute, all occur in series 2, whereas the others are placed in series 3. Of the latter, the sole effective substrate was holocellulose for *Botrytis cinerea* 162 and *Aspergillus unguis* 143, and alkali jute for *Chaetomium brasiliense* 117, *Aspergillus chevalieri* 88, and *Penicillium* sp. 91. The titers were usually rather low.

The organisms in series 4 (15 strains, 12 species, 11 genera) which did not form cellulase on any of the four substrates included no *Aspergillus* species and only one *Penicillium* species (*P. tardum*). The other species included were *Actinomyces* sp., *Botrytis alli*, *Cephalosporium* sp., *Chaetomium elatum*,

C. trigonosporum, *Cunninghamella* sp., *Heterosporium* sp., *Metarrhizium anisopliae*, *Paecilomyces varioti*, *Pullularia pullulans*, and *Syncephalastrum* sp. Moderate to heavy growth was shown only by *Paecilomyces varioti* on holocellulose and *Actinomyces* sp., *Chaetomium elatum*, and *Metarrhizium anisopliae* on jute. Although frequently found on mildewed jute materials, *P. varioti* had previously shown little strength loss of jute cloth. However, this loss had been 28% and 32% for *C. elatum* and *M. anisopliae* respectively (4).

The fact that holocellulose and alkali jute proved to be better substrates for growth and enzyme production than α-cellulose and jute suggested that a marked effect in this respect was exerted by the hemicellulose in jute, when freed of its association, chemical or physical, with the other constituents. This point was further studied with selected fungi, using completely isolated hemicellulose and filter paper. Jute α-cellulose was used as a control. In the control flasks for growth, 0.1 g hemicellulose and 0.4 g α-cellulose from jute were used singly; in the experimental flasks 0.05 g hemicellulose and approximately 0.35 g filter paper were taken together. Most of the fungi were naturally selected from the group not producing enzyme on filter paper or jute (series 3), but six strong cellulose decomposers (series 1) were also included. Incubation was for 10 days at 30° C.

Of the 21 organisms (16 species, 3 genera) in the first group, the two belonging to the *Aspergillus niger* species group seemed to prefer hemicellulose alone to hemicellulose + cellulose (these may perhaps be called true hemicellulose decomposers), while eight fungi (six species, two genera) showed just the opposite preference. These are arranged in Table IV in order of the PF titer of their hemicellulose enzyme. The utilization of cellulose was apparently not stimulated by hemicellulose in the rest of the organisms, namely *Penicillium simplicissimum* 4, *P. implicatum* 53, *P. purpurogenum* 134, *P.* spp. 40 and 135, *P. wortmanni* 126.78, 130.63, and 130.64, *P. fellutanum* 7, *P. variabile* 34, and *P. steckii* 137. It is interesting to note that the first five of these species gave no PF titer from cellulose + isolated hemicellulose or the latter alone, although they had previously produced enzyme from holocellulose. There was generally some correlation between growth and enzyme production, but again there were instances, particularly with hemicellulose, where little enzyme was produced corresponding to heavy growth.

All six species of pure cellulose decomposers (series 1) gave heavy growth on all substrates. The effect of hemicellulose on the utilization of cellulose for cellulase production was very striking with the first three of these species. On the other hand, while on *Aspergillus fumigatus* 14 and *A. ustus* 29, hemicellulose seemed to have no effect, it appeared to exert a markedly inhibitory influence on enzyme production by *Penicillium* sp. 98, though not on its growth.

Although evidence was obtained that jute hemicellulose has a favorable effect on cellulase production from pure cellulose, the alkali jute studied, which has generally proved the most suitable of the four substrates, was prepared by a treatment (with 1% NaOH) which removes a large proportion

(38%) of the hemicellulose (14). However, 9.3% NaOH is known to remove the maximum amount of hemicellulose from holocellulose (20). Alkali jute prepared by 9.3% NaOH under similar conditions was compared with the previous product, using 17 fungi which had given particularly high titers on the latter.

TABLE IV

Effect of hemicellulose

Serial No.	Species and ref. No.	Enzyme titer					
		Hemicellulose (jute)		α-Cellulose (jute)		Hemicellulose + filter paper	
		PF	SC	PF	SC	PF	SC
	A. Fungi giving little or no enzyme on jute and filter paper (series 3)						
1	*Aspergillus atropurpureus* 92	2.30	3.90	0.00	0.00	0.50	1.70
2	*Aspergillus niger* 3	1.10	4.30	0.00	0.00	0.70	2.60
3	*Penicillium wortmanni* 126.24	0.40	1.70	0.00	0.00	0.70	1.50
4	*Penicillium wortmanni* 130.30	0.30	0.90	0.00	0.00	0.70	1.40
6	*Penicillium funiculosum* series 150	0.30	0.50	0.00	0.20	0.60	1.10
8	*Penicillium citrinum* 37	0.20	0.80	0.10	0.50	0.40	1.10
9	*Penicillium vermiculatum* 77.62	0.20	0.70	0.00	0.05	0.80	2.30
16	*Scopulariopsis* sp. 120	0.00	0.00	0.00	0.00	0.40	0.70
17	*Penicillium wortmanni* 126.31	0.00	0.00	0.00	0.00	0.60	1.30
18	*Penicillium rubrum* 127	0.00	0.00	0.00	0.00	0.20	0.80
	B. Fungi giving enzyme on all four substrates (series 1)						
1	*Penicillium variabile* 121	0.80	2.80	2.90	6.10	3.80	8.20
2	*Penicillium janthinellum* series 153	0.20	0.80	2.60	6.10	6.10	10.10
3	*Aspergillus terreus* 6.2	0.20	0.30	2.60	6.10	6.20	9.60
4	*Aspergillus fumigatus* 14	0.10	0.30	2.30	4.80	2.20	5.00
5	*Penicillium* sp. 98	0.10	0.30	3.05	7.80	0.40	1.10
6	*Aspergillus ustus* 29	0.10	0.20	2.10	4.60	2.00	4.80

In most cases substantially higher PF titers were given by the filtrates obtained from the standard alkali jute, containing relatively greater hemicellulose, the reverse being the case only with *Penicillium brefeldianum* and two strains of *P. wortmanni*; the latter tendency was seen more often in the SC titers. On the whole it seems that retention of hemicellulose in alkali jute is favorable for enzyme production.

Jute hemicellulose comprises, apart from polyuronides, a pentosan fraction composed of xylose, arabinose, and rhamnose units, and a hexosan fraction containing glucose, galactose, and mannose (17). In order to compare the effect of these sugars with that of hemicellulose, free xylose (Merck), arabinose (Merck), and glucose (BDH, Analar) were similarly tested in association with filter paper for enzyme production. The quantity was 0.1 g per flask when alone and 0.05 g when with filter paper. Starch, often used as a sizing material with cellulosic fibers, was also tested in the form of a commercial maize starch, taken in 0.075-g quantities per flask when alone and 0.05 g when with cellulose. Incubation was for 10 days at 30° C in all cases.

The fungi tested were selected from Table IV. With the non-cellulose decomposers (series 3), in no case was there any growth or enzyme production on

TABLE V

Effect of sugars and starch

Species and ref. No.	Enzyme titer									
			Filter paper with added:							
	Filter paper alone		Starch		Xylose		Arabinose		Glucose	
	PF	SC	PF	SC	PF	SC	PF	SC	PF	SC
A. Fungi giving little or no enzyme on jute and filter paper (series 3)										
Penicillium wortmanni 126.78	0.00	0.00	4.10	11.60	4.10	11.50	1.60	6.30	3.30	11.00
Penicillium wortmanni 126.31	0.00	0.00	3.80	9.80	3.10	11.10	0.30	2.80	2.60	8.60
Penicillium wortmanni 126.24	0.00	0.00	3.60	6.70	2.80	10.60	1.80	6.40	2.80	8.70
Penicillium rubrum 127	0.00	0.00	1.60	3.00	2.40	4.60	1.20	3.30	1.20	2.60
Penicillium wortmanni 130.30	0.00	0.00	1.00	4.50	1.00	6.80	0.40	2.70	0.10	1.80
Aspergillus atropurpureus 92	0.00	0.00	0.70	3.60	0.20	2.60	0.10	0.50	0.30	1.50
Penicillium wortmanni 130.63	0.00	0.00	0.50	5.40	1.10	6.30	0.20	2.80	0.30	3.30
Penicillium wortmanni 130.64	0.00	0.00	0.10	2.50	0.10	0.60	0.00	0.00	0.00	0.00
Aspergillus niger 3	0.00	0.00	0.00	0.00	0.10	2.20	0.00	0.00	0.10	0.80
Scopulariopsis sp. 120	0.00	0.00			0.00	0.00	0.00	0.00	0.00	0.00
Penicillium steckii 137	0.00	0.00			0.00	0.30	0.00	0.00	0.00	0.00
B. Fungi giving enzyme on all four substrates (series 1)										
Penicillium sp. 98	3.10	7.80	1.20	2.40	0.00	1.40	0.80	2.80	0.10	0.50
Penicillium janthinellum series 153	2.10	5.60	0.70	3.00	0.70	3.10	0.80	3.30	0.00	0.00
Penicillium variabile 121	3.10	7.80	0.70	2.40	0.80	3.30	1.90	5.80	1.10	2.70
Aspergillus terreus 6.2	2.80	6.80	0.20	1.80	0.00	0.00	0.00	0.20	0.00	0.00
Aspergillus fumigatus 14	1.60	5.40	0.00	0.60	0.20	0.80	0.00	0.40	1.80	5.60
Aspergillus ustus 29	1.00	1.80	0.00	0.30	0.25	1.60	1.10	3.10	0.00	0.20

filter paper or starch alone, and on the sugars the only instance of a titer (PF 0.20 ml, SC 0.40 ml) was with xylose, as a medium for *Aspergillus niger* 3. With the pure cellulose decomposers (series 1), again xylose was the only sugar producing some enzyme when alone, the organisms concerned being *Aspergillus fumigatus* 14 and *Penicillium janthinellum* 153, and the titers low (PF 0.10 ml and SC 0.30 ml, in both cases). The rest of the data are given in Table V, the organisms being arranged in order of the PF titer of the filtrate from the starch medium.

It is seen that with nearly all the so-called 'non-cellulolytic' species growth on cellulose and cellulase production could be stimulated by simple sugars, usually to a greater extent than by isolated hemicellulose. In this respect xylose was the most effective of the three (xylan constitutes nearly 50% of jute hemicellulose), and the 126 group of *Penicillium wortmanni* most sensitive among the organisms. As a supplement, starch gave titers of the same order as xylose, although *Aspergillus niger* 3 remained completely unaffected. The stimulation may however be due to contaminating growth substances which, as will be seen, sometimes have a marked effect. Again *Penicillium wortmanni* 126 gave the highest titers, the No. 130 strains responding much more feebly; reaction to starch or xylose as supplements is another feature which makes a distinction between the two groups of this species (2), although hemicellulose does not show any such difference. Sharply contrasted was the behavior of the strong cellulose decomposers, there being an inhibition of cellulase production almost without exception, suggesting that the added carbohydrates exerted a protective effect on cellulose.

The effect of supplementation with micronutrients was next studied in similar experiments with the same species and three others from series 2 (two strains of *Memnoniella echinata* and one of *Penicillium vermiculatum*) which had been seen to be responsive to jute but not to filter paper. In one set, 0.01% yeast extract was added to the medium. A mixture was also used, comprising, per flask, Zn and Mn at 10 p.p.m. and Fe at 100 p.p.m. (all as metal) and 10 μg thiamin and 1.0 μg biotin, the two vitamins previously found to have a remarkable effect on the growth and reproduction of several species capable of strongly decomposing jute and cellulose (9, 10).

The results, given in Table VI, show that both supplements stimulated enzyme production by fungi belonging to series 1 (cellulose decomposers), as well as by the ones selected from series 2; of these latter instances, two are accounted for by the well-known biotin deficiency of *Memnoniella echinata* (10).

As judged from the effect on the cellulose decomposers, the action of the two supplements seemed to be similar in nature; notable, however, was the reaction of *Penicillium* sp. 98 whose SC titer (but not PF titer) showed a marked increase with yeast extract but was unaffected with vitamin–salts.

The marked growth-stimulating effect of jute extract has been observed in our previous studies (5). In the present work, for several fungi jute had proved a better medium for growth and enzyme production than filter paper and even holocellulose; in some instances, even alkali-treated jute proved

less favorable than jute (Table I). This suggested that for these fungi the micronutrients present in jute were of primary importance, and this point was further studied. The relevant fungi were grown on filter paper with the hot-water extract of 5.0 g jute per 100 ml of the final medium.

The results given in Table VII show severalfold increase in enzyme production by all the fungi with jute extract. Growth was heavy in all cases, but even the fungi giving heavy growth without jute extract registered appreciable increases in enzyme production. Of the nine species tested, six were *Penicillium* and these reacted most markedly.

TABLE VI

Effect of micronutrients

Species and ref. No.	Enzyme titer: Filter paper with yeast extract PF	Filter paper with yeast extract SC	Filter paper with vitamins and salts PF	Filter paper with vitamins and salts SC	Filter paper alone PF	Filter paper alone SC
A. Fungi giving little or no enzyme on jute and filter paper (series 3 and 2)						
Penicillium wortmanni 126.78	0.00	0.00	0.00	0.00	0.00	0.00
Penicillium wortmanni 126.31	0.00	0.00	0.00	0.00	0.00	0.00
Penicillium wortmanni 126.24	0.00	0.00	0.00	0.00	0.00	0.00
Penicillium rubrum 127	2.30	5.60	0.10	2.10	0.00	0.00
Penicillium wortmanni 130.30	0.00	0.00	0.00	0.00	0.00	0.00
Aspergillus atropurpureus 92	0.00	0.00	0.00	0.00	0.00	0.00
Penicillium wortmanni 130.63	0.00	0.00	0.30	1.80	0.00	0.00
Penicillium wortmanni 130.64	0.00	0.00	0.00	0.00	0.00	0.00
Aspergillus niger 3	0.00	0.00	0.00	0.00	0.00	0.00
Memnoniella echinata 73	0.20	1.20	0.40	2.30	0.00	0.00
Memnoniella echinata 73.1	0.40	1.60	0.40	1.80	0.00	0.00
Penicillium vermiculatum 77.10	0.00	0.00	0.30	1.20	0.00	0.00
B. Fungi giving enzyme on all four substrates (series 1)						
Penicillum sp. 98	1.40	7.10	0.90	1.50	0.80	1.50
Penicillium janthinellum series 153	2.80	8.90	3.20	8.10	2.50	6.55
Penicillium variabile 121	4.40	9.60	4.20	10.00	2.90	8.50
Aspergillus terreus 6.2	5.30	10.10	4.60	9.80	3.00	7.50
Aspergillus fumigatus 14	2.00	8.10	1.80	7.10	1.55	6.00
Aspergillus ustus 29	3.20	8.60	1.20	3.10	0.75	2.30

TABLE VII

Effect of jute extract

Species and ref. No.	Enzyme titer: Filter paper with jute extract PF	Filter paper with jute extract SC	Filter paper alone PF	Filter paper alone SC	Growth: Filter paper with jute extract	Growth: Filter paper alone
Penicillium vermiculatum 77.29	6.20	10.90	0.30	1.60	4	3
Penicillium oxalicum 96	3.10	6.60	0.25	1.00	4	4
Penicillium vermiculatum 77.10	2.60	6.90	0.00	0.00	4	0
Penicillium brefeldianum 146	0.90	3.00	0.50	1.50	4	1
Penicillium verruculosum 158	0.50	2.00	0.05	0.30	4	4
Penicillium cyaneum 124	0.50	1.60	0.10	1.10	4	1
Chaetomium funicola 112	0.40	1.20	0.00	0.00	4	4
Heterosporium sp. 104	0.40	0.90	0.20	0.60	4	4
Chaetomium cancroideum 108	0.20	0.30	0.00	0.10	4	4

Discussion

The most important result of this study is the finding that although the enzyme capable of decomposing pure cellulose is produced by a comparatively limited number of fungi when grown on pure cellulose, most of the organisms tested produced the same enzyme when grown on mixed cellulosic substrates. Of these latter, the stimulatory effect of untreated jute fiber was shown to be primarily due to water-soluble micronutrients. These were largely removed in the preparation of alkali jute and holocellulose; these treatments also remove some and all lignin respectively. Since the removal of lignin from jute and the presence of hemicellulose have previously been shown to exert a favorable effect on growth and cellulose decomposition by living fungi, it is natural that holocellulose should produce more enzyme than jute or that alkali jute with a greater retention of hemicellulose should prove more favorable. What is more difficult to understand is the increase in susceptibility of jute on alkali treatment. This may be due to a breakdown of chemical bonds, such as the ester bonds postulated between polyuronic acid and lignin (20), and between acetic acid and the carbohydrate constituents, particularly in hemicellulose (8), both of which are said to be completely broken down on 1% alkali boiling (liberating chemical groups preferred by microorganisms).

However, when holocellulose is compared with isolated hemicellulose plus filter paper as substrate, it is found that less enzyme was produced on the latter by fungi that normally do not yield cellulase from pure cellulose. Since jute α-cellulose by itself is somewhat less easily decomposed by fungi than filter paper (5), this seems to be an effect of the complete isolation of hemicellulose. Thus, all that can be suggested on present evidence—and present knowledge on the chemistry and physics of hemicellulose and its modes of association—is the broad generalization that the most favorable state, for most at least of the so-called 'non-cellulolytic' fungi, is a partial loosening, and not complete separation, of this constituent. On the other hand, it seems that even a loose association may be unfavorable to some of the cellulose-decomposing fungi, since four out of six such species tested definitely preferred free hemicellulose with cellulose to holocellulose. This appears to be a simple additive effect of cellulase produced from hemicellulose and cellulose.

With added sugars or starch, some stimulation was nearly always found with fungi unable to produce cellulase from pure cellulose, and often sugars, particularly xylose, proved to be better than free hemicellulose. This stimulation is more difficult to explain, since none of these carbohydrates produced cellulase by themselves, as did hemicellulose. However, the strains of one species only, namely, *Penicillium wortmanni*, formed most of the organisms preferring sugar; more data are required on the subject of relative preference for sugars or hemicellulose and the mechanism of stimulation of enzyme production by these two groups of substances. It also remains to be explained why sugars and starch did not in fact depress enzyme production with these species, as they did with the strong cellulose decomposers, owing, obviously, to a sparing effect on cellulose. This phenomenon, as well as the effect of free

and attached hemicellulose discussed earlier, may indicate a rather fundamental metabolic difference between species capable and incapable of producing cellulase from pure cellulose.

The effect of micronutrients as supplements seemed to be of a different nature from that of hemicellulose or sugars. While most of the species tested were favorably affected by the latter, of the species unable to produce enzyme from pure cellulose, only a few were stimulated by yeast extract or vitamin – trace elements mixture; the three strains of *Penicillium wortmanni* 126, one of the species strongly influenced by hemicellulose and sugars, remained totally unaffected, while *Penicillium vermiculatum* 77.10 was induced to grow and produce enzyme. The effect of jute extract, tested on species showing a preference for raw jute over alkali jute, was more striking, which is not surprising, since it has been shown to contain many important organic and inorganic micronutrients (3, 10) and to have a growth-stimulatory effect on a wide variety of fungi (3, 5, 10).

Several fungi have been mentioned, when analyzing Table III, which show a wide discrepancy between previous strong in vivo activity and present poor cellulase production. A striking example of loss in enzyme production capacity within a comparatively short period is provided by strain No. 6.2 of *Aspergillus terreus*, which had given 257% and 314% higher PF and SC titers respectively, when grown on filter paper, soon after isolation from a damaged jute bag. Such a progressively acquired deficiency can sometimes be explained on the basis of development of need for the exogenous supply of organic growth factors, as strikingly illustrated in the case of *Memnoniella echinata* 70, which lost its original capacity to grow on and decompose filter paper due to acquired biotin deficiency (10). However, some of the instances mentioned above appear to be an effect of time and subculturing not related to a gradual development of micronutrient deficiency; the organism may be one, like the chaetomiums, with a fundamental discrepancy between cellulose decomposition and cellulase production, as determined by our present methods.

This latter type of discrepancy presents another interesting problem. No question of degeneration arises, since heavy growth on, and decomposition of, pure cellulose and poor enzyme production are displayed simultaneously. Among such species are two of the most well-known cellulose decomposers, *Chaetomium globosum* and *Myrothecium verrucaria*, and of these the latter has so far been the most widely used species for enzyme studies. Species like *Penicillium variabile* and *Aspergillus terreus*, which have proved so potent in the present investigation, may well be more important in nature than the comparatively rare *Myrothecium verrucaria*.

The generally higher titers given by swollen cellulose, as compared with filter paper, are to be expected, since it contains a bigger amorphous fraction (26). However, the trends of rise or fall of the two titers were not always strictly parallel, although such indirect data with impure enzymes cannot probably be taken as confirmation of multiple components in fungal cellulase.

What seems certain is that on a suitable substrate a fungus previously regarded as 'non-cellulolytic' may produce enzymes capable of degrading relatively complex forms of cellulose, if not native cellulose.

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A MICROBIOLOGICAL ASSAY FOR THE QUANTITATIVE DETERMINATION OF GLYCEROL[1]

L. EIDUS, B. B. DIENA, A. C. MANIAR, AND L. GREENBERG

Abstract

A microbiological assay has been developed using the techniques of assay for antibiotics. Metal cylinders were placed on plates prepared by overlaying a thin layer of seed agar, inoculated with a test organism, on a base of nutrient agar. Appropriate dilutions of a standard glycerol solution and of the glycerol preparations being tested were added to the proper cylinders. The plates were incubated and the zone sizes around the cylinders measured. The potency of the preparations was determined by comparing the average zone size of the unknown preparations with that of the standard. The method proved to be simple to perform, and in the absence of interfering substances, yielded essentially the same results as chemical methods. A number of carbohydrates—glucose, laevulose, maltose, mannitol, mannose, saccharose, and trehalose—interfered with assay results so that the method cannot be used for glycerol determinations in mixtures where they are present. No interference, however, was noted with *d*-arabitol and erythritol, which were found to interfere with the chemical assay.

Introduction

During the course of studies on the antibiotic-producing properties of mycobacteria, it was noted that glycerol had a marked inhibitory effect on the growth of a number of our test organisms. The mycobacteria, particularly strains isolated from human sources, can metabolize glycerol. The medium employed for their growth was Sauton's which contains 6% glycerol and the amount left following 4 to 8 weeks' incubation varied with different strains. Using the cylinder plate method, it was found that the zone of inhibition around the cup varied directly with the amount of glycerol remaining in the culture fluid. These findings were confirmed using pure glycerol (see Figs. 1, 2, 3) and further, it was found that the test organism was inhibited by levels of 20 mg or higher. This suggested the possibility of a quantitative microbiological assay and the method developed for this purpose is described here.

Procedures

The procedures used were similar to those of the cylinder plate assays for antibiotics described by Grove and Randall (1). The test organism was *Staphylococcus aureus* strain 8178. Three commercial media (Baltimore Biological Laboratories) were used.

Ten milliliters of antibiotic base agar (B.B.L.) are poured into a petri dish and allowed to harden. Following this, 4 ml of inoculated antibiotic seed agar are poured evenly over the base agar in a thin layer and allowed to harden for 15 minutes. The seed agar is prepared by inoculating melted agar cooled to 48° C with 4 ml of broth culture for every 150 ml. The broth culture is prepared by growing the test organism in antibiotic assay broth for 36 hours at 37° C. After the seed agar has hardened, two cylinders are placed evenly

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Contribution from the Biologics Control Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

apart on the plate. One cylinder is filled to the brim with a standard solution containing 50 mg/ml of the glycerol and the other is filled to the same level with the unknown solution. Six plates are used for each assay and these are incubated at 37° C for 16 to 18 hours. The average zones of inhibition are calculated and the unknown is compared with the standard against a standard curve which is prepared in the following manner.

Forty-two plates are prepared as described above. Dilutions of a standard glycerol solution are prepared to contain 20, 30, 40, 50, 60, 70, and 80 mg/ml, respectively. Six plates are used for each dilution. Following incubation, the average zone size is determined for each level and a standard curve is prepared on semilog graph paper with the zone size shown on the abscissa, the strength in mg/ml as the ordinate.

Method of Calculation

The size of zone for the standard preparation is determined and compared with the standard curve. If the zone size for the standard in the assay (50 mg/ml) is identical with that shown on the standard curve for 50 mg/ml, the potency of the unknown may be determined directly from the graph. If the zone size differs from that shown on the standard curve, the readings in milligrams (the ordinate on the graph corresponding to the zone sizes found in the assay) for both the standard and unknown are taken from the standard curve and the potency may be calculated as follows:

$$\text{Potency (mg/ml)} = \frac{\text{reading of the unknown in mg}}{\text{reading of the standard in mg}} \times 50.$$

Results of Assays

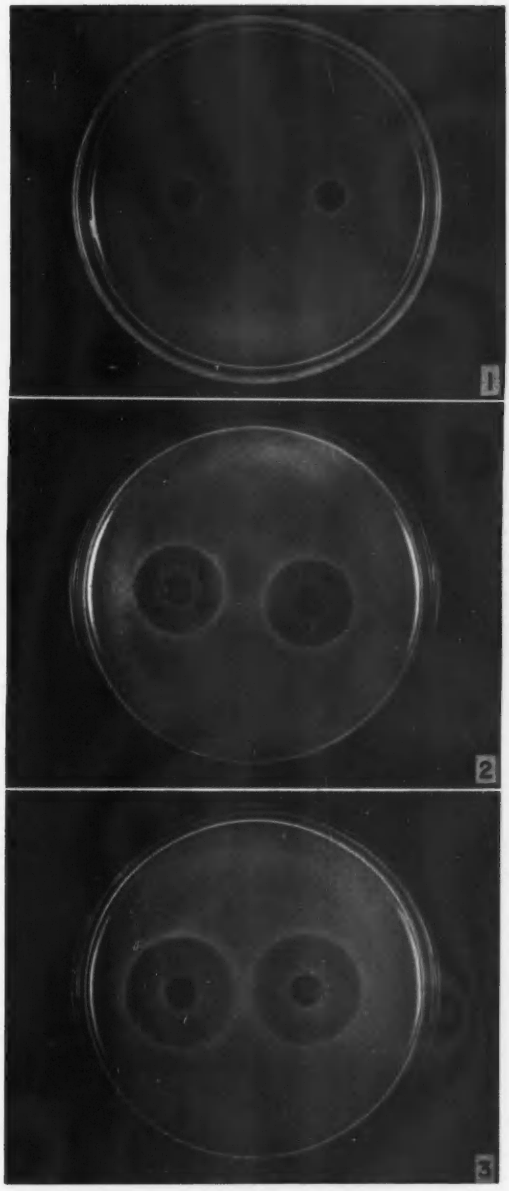
Twelve solutions of glycerol of known concentration were prepared and assayed microbiologically by the method described, and chemically by Neish's technique (2). The results are shown in Table I. The two methods yielded

TABLE I

A comparison of the microbiological assay and chemical method for the determination of glycerol

Amount of glycerol in solution, mg/ml	Assay results	
	Microbiological, mg/ml	Chemical, mg/ml
20	21	21
20	22	20
30	34	30
30	33	30
40	40	43
40	42	41
50	50	55
50	52	50
60	60	62
60	65	62
80	76	80
80	81	80

PLATE I



FIGS. 1–3. The bacteriostatic effect of glycerol to *Staphylococcus aureus* strain 8178.
Fig. 1. Control—no glycerol. Fig. 2. Glycerol—30 mg/ml. Fig. 3. Glycerol—50 mg/ml.

similar results, and appear to have a similar degree of precision. In the microbiological assays the greatest variation in results from the known concentration of glycerol was 12% and three agreed exactly with the standard. In the chemical test the greatest variation was 10% and six agreed exactly with the standard.

Microbiological assays for glycerol were also carried out on mixtures containing glycerol and other carbohydrates (reagent grade). Sixteen carbohydrates were tested. The results are shown in Table II. No interference in assay

TABLE II

Results of assays with mixtures containing glycerol and other carbohydrates

Mixtures containing 50 mg/ml glycerol and 50 mg of carbohydrate shown below	Microbiological assay, mg/ml
Arabinose	51
d-Arabitol	50
Dulcitol	50
Erythritol	50
Galactose	52
Inositol	50
Rhamnose	49
Sorbitol	48
Xylose	49
Mannitol	62
Dextrose	73
Laevulose	67
Maltose	61
Mannose	75
Saccharose	72
Trehalose	67

TABLE III

A comparison of the microbiological assay and chemical methods in the determination of glycerol in culture fluids of mycobacteria

Strain	Microbiological assay (glycerol in mg/ml)	Chemical assay (glycerol in mg/ml)
Human 1	18.1	16.8
Human 2	20.2	18.0
Human 3	27.5	30.2
Atypical 1	20.7	23.8
Atypical 2	19.8	17.1
Atypical 3	22.7	20.9
Bovine 1	66.6	61.4
Bovine 2	7.3	8.0
Bovine 3	14.5	11.2
Avian 1	3.1	5.0
Avian 2	0	0
Avian 3	0	0
Saprophyte 1	0	0
Saprophyte 2	9.6	11.1
Saprophyte 3	20.9	20.9

results was found with arabinose, *d*-arabitol, dulcitol, erythritol, galactose, inositol, rhamnose, sorbitol, or xylose, but differences of 20% or greater in assay results were noted with glucose, laevulose, maltose, mannitol, mannose, saccharose, and trehalose.

A series of tests were carried out on culture fluids following the growth of a number of mycobacteria. Both microbiological and chemical assays were performed. The results of assays from 16 cultures (three strains from each of five separate species selected at random) are shown in Table III. Here, too, the methods yielded similar results.

Discussion

A microbiological assay for the quantitative determination of glycerol using *Staphylococcus aureus* strain 8178 has been described. The techniques which are relatively simple are similar to those of the cylinder plate bio-assay for antibiotics (1). A major difference between the assays for glycerol and those for antibiotics is that for the former the zones surrounding the cylinder are not as clear as they are on the antibiotic plates. On the glycerol plates, growth of the organism takes place right to the cylinder but the density of the growth adjacent to the cylinder is very much reduced giving the effect of a distinct circular clearing with a well-marked circumference (see Figs. 2 and 3).

The seed agar used contains 0.15% yeast extract and 0.1% dextrose. In our experience, the omission of the yeast extract and the dextrose from this medium will not influence the size or clarity of the zone. Increasing the dextrose content beyond 0.1%, however, will detract from the clarity of the zones.

The method for preparing a standard curve is also described. In this study, a standard curve was prepared for each assay. Our experience, however, has shown that this may not be necessary. The curves have shown remarkably little change during the period of this study and the results indicate a curve may be used for at least one month.

To determine the specificity of the microbiological method, or whether the presence of other carbohydrates would interfere with assay results, a number of assays were carried out on mixtures containing glycerol and other carbohydrates. The effect of the presence of 16 different carbohydrates was studied in this manner. A number (see Table II) did not interfere with the assay results, but with others there was marked interference. The method, therefore, has limited value for the determination of glycerol in mixtures containing any of the latter group.

A series of tests were performed on culture fluids following the growth of mycobacteria. The results of both the microbiological and chemical assays were similar in each case indicating that the fermentation by-products present in the fluid following a 4- to 8-week growing period did not interfere with the assays.

A comparison between the microbiological and chemical assays for glycerol determination has shown that there was very little difference in results between the two methods. The microbiological method has an advantage in that, once

the techniques are established, it is a simpler test to perform and that relatively large numbers of assays can be carried out in a short period of time with a minimum amount of work. The limitation of the microbiological technique is that it cannot be used in the presence of certain carbohydrates. The chemical assay is also limited in this regard since interference has been noted with *d*-arabitol and erythritol (3). The chemical assay has an advantage in that it can measure much smaller amounts of glycerol. At present, there are relatively few methods available for assaying glycerol. The microbiological technique could prove a very useful supplement to existing methods.

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UTILIZATION OF CARBOHYDRATES AND AMINO ACIDS BY MICROCOCCUS RADIODURANS[1]

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Abstract

The nutrition and metabolism of a recently isolated *Micrococcus* species resistant to doses of gamma radiation as high as 6×10^6 r.e.p. were studied by manometric and radioactive tracer techniques. Methionine, the only amino acid shown to be essential in a chemically defined medium, appears to be rapidly incorporated into the cells. DL-Glutamic acid is readily metabolized, the D isomer apparently after an initial lag. Among the simple carbohydrates, fructose, glucose, and glycerol are readily utilized. The operation of the tricarboxylic acid cycle is suggested by the oxidation of certain TCA intermediates. The prompt conversion of C-1 of gluconate to CO_2, in the presence of glucose, may indicate that a C_1–C_5 cleavage pathway is operative for catabolism of glucose in this organism.

Introduction

During studies on the effect of gamma radiation on sterilization of food products, a bacterial species (tentative nomenclature: *Micrococcus radiodurans*)[3] was isolated from meat which had been exposed to dosages of 2×10^6 and 3×10^6 roentgen equivalent physical (r.e.p.). The presence of the radiation-resistant organism was subsequently confirmed by culturing the organism in various liquid and solid media and then irradiating these at two radiation sites which included cobalt 60 and atomic fission by-products. Exposure to various dosages demonstrated that this microorganism, a nonsporeforming, pink-pigmented tetracoccus, survives 4×10^6 r.e.p. in ground meat or tryptone glucose yeast extract broth (TGY), and up to 6×10^6 r.e.p. on TGY agar slants. In view of the unique ability of this micrococcus to withstand a radiation dose as high as 6×10^6 r.e.p., it seemed desirable to examine its nutritional characteristics as well as physiological behavior.

In the present work, a chemically defined medium was devised to support optimum growth of *M. radiodurans*. The utilization of simple carbohydrates, intermediates of the tricarboxylic acid (TCA) cycle, and amino acids by this organism was studied by means of the manometric and radiorespirometric techniques. The roles played by various substrates in cellular respiration and biosynthesis were also examined.

Materials and Methods

Organism

The strain of *Micrococcus radiodurans* used was first isolated in the Department of Bacteriology, Oregon State College (1), from irradiated meat samples.

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Contribution from the Departments of Bacteriology and Chemistry, Oregon Agricultural Experiment Station. Technical Paper No. 2003. This investigation was supported in part by research grant E-1880 from the National Institutes of Health, Public Health Service.
[2]Present address: Department of Bacteriology, University of Washington, Seattle, Washington.
[3]This organism has been deposited in the American Type Culture Collection.

Medium

For manometric studies on resting cells the organism was grown in TGY broth (0.5% tryptone, 0.1% glucose, 0.1% yeast extract). However, since a chemically defined medium permitting optimum growth was more desirable for metabolic studies employing radioactive tracers, a synthetic medium (Table I) was developed which supports luxuriant growth of one of the strains within 24 hours.

Manometric Studies on Resting Cells

The cells were grown in 50 ml of TGY medium in 250-ml bottles on a rotary type shaker at 30°–34° C for 24 hours. The cells were collected from the medium by centrifugation at 3000 r.p.m., washed twice with 0.05 *M* phosphate buffer at pH 7, and then were resuspended in buffer to the desired concentration by means of optical density determinations. After depleting the cells by shaking for 1 hour, 2.5 ml of the cell suspension (containing approximately 3 mg dry weight of cells) were introduced into each Warburg flask. The substrates were dissolved in 0.066 *M* phosphate buffer adjusted to pH 7 and 0.5 ml of the substrate solution (usually containing 10 μmoles) was placed in the side arm of each flask. For measurement of endogenous respiration 0.5 ml of 0.066 *M* phosphate buffer was used in place of a substrate.

The conventional Warburg procedures were employed for manometric studies (5). The reaction vessels were equilibrated for 10 minutes at the water bath temperature of 30° C before substrate addition. The oxygen uptake was measured at intervals (usually 10 minutes) and the experiments terminated after 160–200 minutes.

Manometric Studies on Growing Cells

The cells were cultured in synthetic medium (Table I), centrifuged and washed twice with sterile distilled water, and finally resuspended in the chemically defined medium from which the specific components to be used as test substrates for the experiment were omitted. Two and one-half milliliters of this cell suspension (adjusted by means of optical density measurements to contain approximately 7 mg dry weight of cells) were added to each Warburg flask. The substrates were dissolved in distilled water and 0.5 ml of the substrate solution was introduced into the side arm of each flask. The remaining Warburg procedures were similar to those described for resting cell studies. At the end of the 10-hour experiment, streak plate tests revealed no contamination.

Radiorespirometric Experiments

The procedure was essentially the same as described by Wang *et al.* (6). The cell suspension was prepared as described under manometric studies on growing cells. Ten milliliters of the medium containing a given amount of cells (usually 30 mg, on a dry weight basis) were added to each flask. Tests showed that no serious contamination of the culture developed during an experimental period of 7–10 hours. Following each experiment, cells and

media were separated by centrifugation. Cells were dried at room temperature over P_2O_5 in a vacuum desiccator and weighed. The cells and media were processed for paper chromatographic examination and radioactivity assays.

Radioactive Substrates

D-Glucose-U-C^{14}, D-fructose-U-C^{14}, potassium gluconate-1-C^{14}, L-methionine-CH_3-C^{14}, DL-methionine-2-C^{14}, DL-glutamic acid-1-C^{14}, DL-glutamic acid-2-C^{14}, and uniformly labeled yeast hydrolyzate were obtained commercially. All labeled substrates were adjusted to prescribed specific activities prior to administration.

Radioactivity Measurements

The respiratory CO_2 samples obtained during an experiment in the form of barium carbonate were mounted onto aluminum planchets by the centrifugation technique and assayed for radioactivity with a thin window Geiger–Muller (GM) counter. Determinations were made to a standard deviation of 2% and counting data were corrected for background and self-absorption in the conventional manner.

The activities of substrates and media were determined by persulphate combustion according to the method of Katz *et al.* (3) with slight modification. It was found that an additional amount of $AgNO_3$ was required to combine with the chloride and possibly other ions present in the acidified medium. Radiochemical assay of the cells was carried out according to the method of Chen and Lauer (2).

Chromatography and Radioautography

Ten milligrams dried cells were hydrolyzed with 1 ml of constant-boiling HCl in a sealed glass tube by autoclaving for 8 hours at 15 lb pressure. Humin was removed by filtration. The filtrate was dried in a vacuum desiccator over KOH and P_2O_5 to remove HCl and H_2O, respectively. For separation of amino acids, aqueous solutions of the filtrate residues were subjected to descending one-dimensional chromatography in an NH_3 atmosphere using 3 parts *sec*-butanol to 1 part 3% NH_4OH as solvent (4). The radioactivity in individual amino acids was examined by conventional radioautographic methods.

Results and Discussion

In Table I is given the composition of the chemically defined medium found to support the growth of *M. radiodurans*. In developing this medium, many combinations of varying amounts of various carbohydrates and amino acids were tested by elimination processes. It was very interesting to note that methionine was the only essential amino acid and that quantities as low as 0.0002% of L-methionine in combination with glucose, a few vitamins, minerals, and nucleic acids were sufficient to meet the minimum demands for growth. However, higher amounts of methionine up to 0.01% were found to give better growth and appeared to increase pigment production. Glutamic acid, though not essential, was found to be quite stimulatory. When 0.05% L-glutamic acid was incorporated in the medium containing 0.001% L-methionine, luxuriant growth was obtained within 24 hours at 30° C.

TABLE I

Composition of the chemically defined medium per 100 ml final volume

Ammonium phosphate (dibasic)	50 mg	Salt solution A	0.5 ml
L-Methionine	1 mg	KH_2PO_4, 25 g	
L-Glutamic acid	50 mg	K_2HPO_4, 25 g	
Biotin	1 μ	Made to 250 ml with dist. H_2O	
Pyridoxine	20 μg	Salt solution B	0.5 ml
Niacin	25 μg	$MgSO_4 \cdot 7H_2O$, 10.0 g	
Thiamin hydrochloride	50 μg	$FeSO_4 \cdot 7H_2O$, 0.5 g	
Glucose	500 mg	$MnSO_4 \cdot 4H_2O$, 0.5 g	
		Made to 250 ml with dist. H_2O	

The pH was adjusted to 6.8 with NaOH

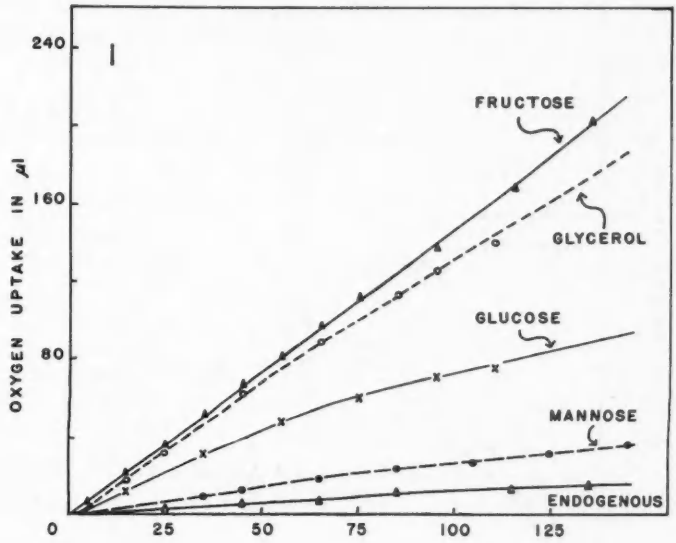
To examine the utilization of various simple carbohydrates, TCA intermediates, and amino acids for the respiratory functions of this organism, a series of manometric experiments were carried out employing resting cells, as shown in Figs. 1 and 2. Of the carbohydrates tested, it was noted that glycerol, fructose, and glucose could be readily utilized whereas mannose, ribose, arabinose, xylose, and gluconate were poor carbon sources. Common TCA cycle intermediates such as succinate, pyruvate, fumarate, and isocitrate were found to be metabolized, although at a rather slow rate, suggesting that the TCA cyclic process probably is operative in this organism.

The requirement of methionine for the growth of *M. radiodurans* is indeed very interesting. Considering the fact that the O_2 uptake curves leveled at comparatively short time intervals proportionate to the concentration of the substrate, and by examining the observed total O_2 uptake at the leveling point (Fig. 2), it appears that this amino acid is rapidly incorporated into the cellular constituents.

The role played by glutamic acid in the growth of *M. radiodurans* was also examined by the manometric method. As shown in Fig. 3, L-glutamic acid was utilized immediately by cells suspended in chemically defined medium. On the other hand, a time lag was observed when D-glutamic acid was used as the test substrate. This finding thus suggests that a mechanism for D-amino acid utilization can be induced to operate in this organism. It is also interesting to note that the O_2 uptake curve of DL-glutamic acid appears to be the summation of the corresponding curves for D- and L-glutamic acid respectively.

FIG. 1. Oxidation of simple carbohydrates by resting cells of *Micrococcus radiodurans* at 30° C. (On this and the following figures, time 0 indicates the time of substrate addition.) (Oxygen uptake in the presence of arabinose, ribose, xylose, and gluconate was not significantly greater than the endogenous.)

FIG. 2. Oxidation of L-methionine, pyruvate, and certain TCA cycle intermediates by resting cells of *Micrococcus radiodurans* at 30° C. (1) L-Methionine (20 μmoles), (2) L-methionine (10 μmoles), (3) L-methionine (5 μmoles), (4) succinate, (5) pyruvate, (6) fumarate, (7) isocitrate, (E) endogenous. (Oxygen uptake in the presence of acetate was not significantly greater than the endogenous.)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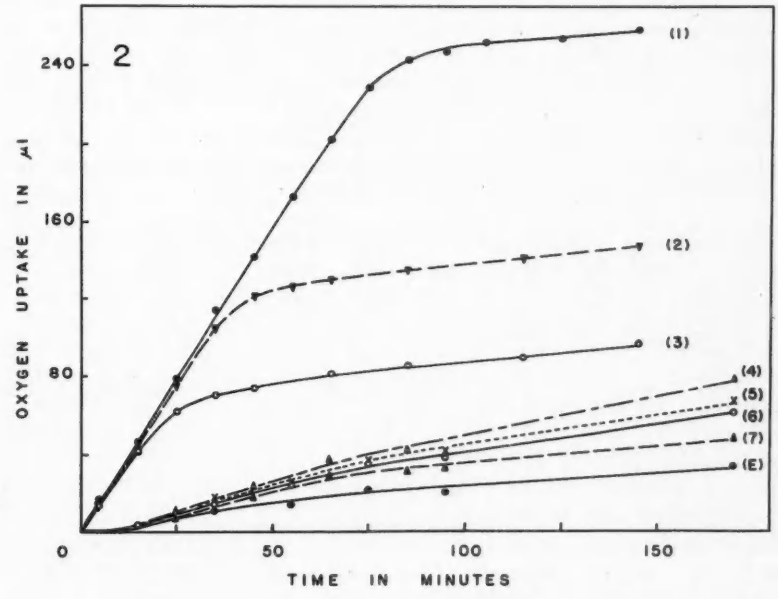
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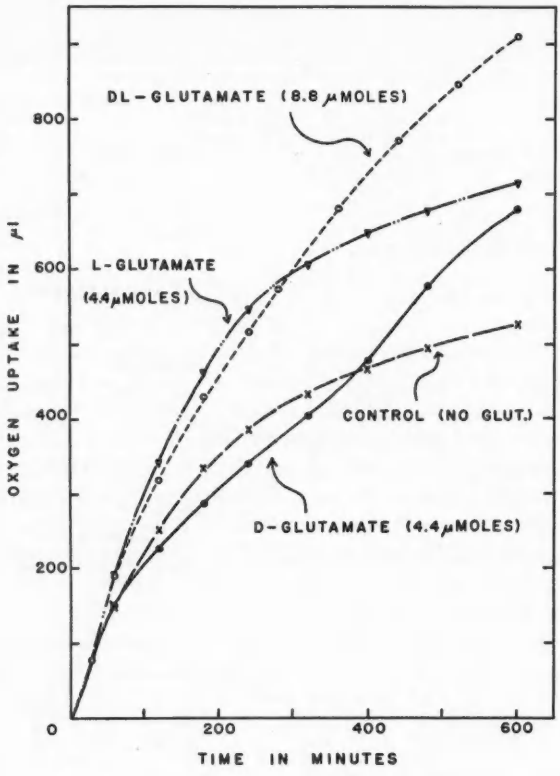


FIG. 3. Oxidation of DL-glutamate, D-glutamate, and L-glutamate by cells of *Micrococcus radiodurans* in the chemically defined medium (glucose omitted).

The utilization of amino acids by *M. radiodurans* was also examined by a series of radiorespirometric experiments, using DL-glutamic acid-1-C^{14}, DL-glutamic acid-2-C^{14}, L-methionine-CH_3-C^{14}, DL-methionine-2-C^{14}, and C^{14} uniformly labeled yeast hydrolyzate as test substrates. The radiorespirometric pattern (time course plot of interval radiochemical recoveries in CO_2) and the time course plot of cumulative recoveries in CO_2 obtained in these experiments are shown in Fig. 4. The over-all radioactive distribution in respiratory CO_2, cells, and incubation medium observed at the end of each experiment are given in Table II.

With DL-glutamic acid-1-C^{14} used as the test substrate, it was found that C-1 of glutamic acid was rapidly converted to CO_2 resulting in an over-all radiochemical recovery of 78%, and as much as 11% of the labeled compound was incorporated into the cells. Similarly DL-glutamic acid-2-C^{14} was also promptly utilized giving rise to a radiochemical recovery in CO_2 of 69%, and 18% of the labeled carbon was incorporated in the cellular constituents.

TABLE II

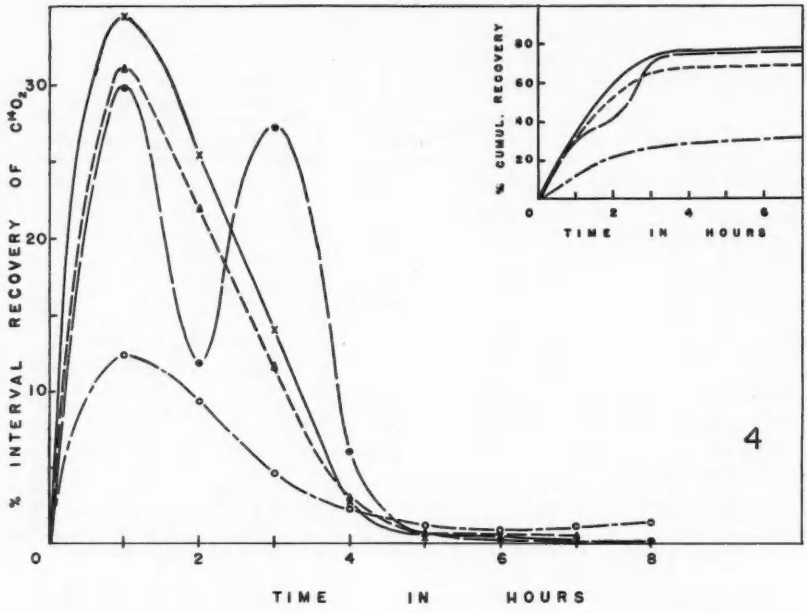
Distribution of substrate activity in *Micrococcus radiodurans* metabolizing C^{14}-labeled amino acids

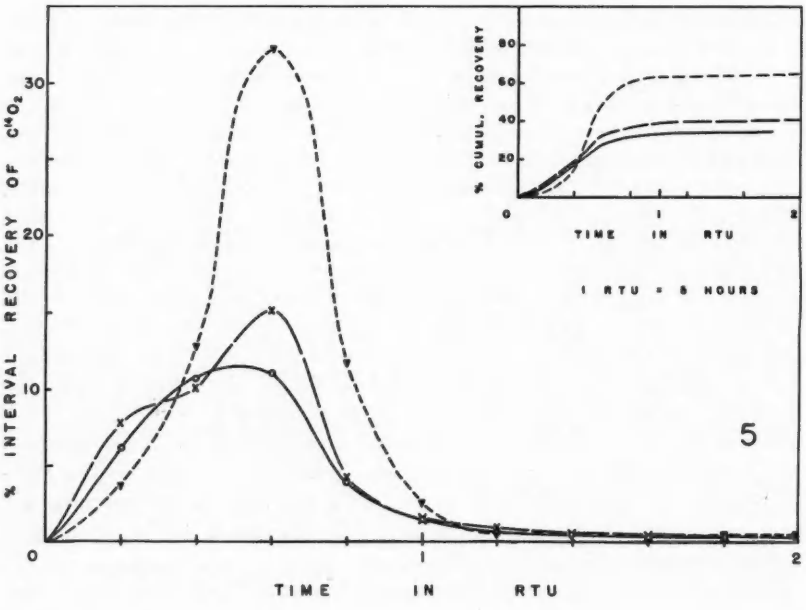
Substrate	Amount, mg	Radio-activity, μc	Radiochemical recoveries, %		
			In CO_2	In medium	In cells
DL-Glutamic acid-1-C^{14} + L-glutamic acid	0.56 + 4.44	5.0	76	14	14
DL-Glutamic acid-1-C^{14} + DL-glutamic acid	0.2 + 4.8	0.5	78	14	11
DL-Glutamic acid-2-C^{14} + DL-glutamic acid	0.2 + 4.8	0.5	69	13	18
L-Methionine-CH_3-C^{14}	0.5	3.2	Trace	35*	32*
L-Methionine-CH_3-C^{14}	0.05	0.32	Trace	13*	30*
DL-Methionine-2-C^{14}	1.00	3.7	Trace	69	18
DL-Methionine-2-C^{14}	0.20	0.74	Trace	27	72
Yeast hydrolyzate-C^{14}	1.25	5	34	20	24

*Low radiochemical recoveries observed with L-methionine-CH_3-C^{14} led us to speculate that the CH_3-C^{14} group of methionine may have been converted to volatile compounds which have been swept out of the incubation system, or to materials in the medium or cells whose radioactivity cannot be determined by the oxidation procedures employed. Further work is underway to determine the fate of the C^{14} which was not recovered.

These findings imply that both D- and L-glutamic acid can be metabolized by this micrococcus, which is in accordance with the observations made in the manometric experiments. As an additional support for the above findings, an experiment was designed using 0.56 mg of DL-glutamic acid-1-C^{14} together with 4.44 mg of L-glutamic acid as carbon source. Data given in Fig. 4 suggests that L-glutamic acid was utilized preferentially to D-glutamic acid as evidenced by the two maxima observed on the radiorespirometric pattern. The radiochemical recoveries in CO_2 from either of these two labeled isomers, D-glutamic acid-1-C^{14} and L-glutamic acid-1-C^{14}, were approximately the same, although the amount of L-glutamic acid present was about 17 times more than that of D-glutamic-1-C^{14}. This observation further suggests that although the basic metabolic mechanism may have been the same the rate of utilization of L-glutamic acid is much faster than that of D-glutamic acid. These results are in good agreement with the observations made in the manometric studies. Although the exact nature of the mechanisms for the utilization of DL-glutamic acid by this micrococcus cannot be determined in the present work, the observed relative rate of $C^{14}O_2$ recovery from glutamic acid-1 and -2-C^{14} indicates that it is probably first converted to alpha-keto glutarate which in turn is metabolized by the TCA processes. The radioautograph of the chromatograms of cell hydrolyzates showed that the bulk of incorporated C^{14} from DL-glutamic-1-C^{14} and DL-glutamic-2-C^{14} appeared to be present in cellular glutamic acid.

With L-methionine-CH_3-C^{14} and DL-methionine-2-C^{14}, it was found that these substrates were rapidly incorporated into the cells with very slight


% INTERVAL RECOVERY OF $C^{14}O_2$
TIME IN HOURS
% CUMUL. RECOVERY
TIME IN HOURS
4


% INTERVAL RECOVERY OF $C^{14}O_2$
TIME IN RTU
% CUMUL. RECOVERY
TIME IN RTU
1 RTU = 8 HOURS
5

C^{14} recovery in the respiratory CO_2. It appears that both the isomers of DL-methionine-2-C^{14} were utilized. Chromatographic and radioautographic examinations of the cell hydrolyzate revealed that a large portion of L-methionine-CH_3-C^{14} probably was incorporated as an intact moiety into cellular constituents.

In an attempt to examine the utilization of various amino acids as carbon sources in a chemically defined medium, a radiorespirometric experiment using C^{14} uniformly labeled yeast hydrolyzate was also carried out. Utilization of amino acids for respiratory functions is evidenced by the prompt appearance of C^{14} in CO_2, resulting in an over-all radiochemical recovery of 34% at the end of the experiment and 24% incorporated into the cellular constituents. These findings implied that amino acids other than glutamic acid and methionine can also be utilized as carbon sources. Paper chromatographic and radioautographic examination of hydrolyzed *M. radiodurans* cells further revealed the presence of the following labeled amino acids in the approximate order of their radioactivities: leucine, isoleucine, tyrosine, methionine, and phenylalanine. Further work is being carried out to elucidate the presence of other amino acids.

Radiorespirometric studies on the utilization of glucose-U-C^{14}, fructose-U-C^{14}, and potassium gluconate-1-C^{14} (in the presence of unlabeled glucose) (Fig. 5; Table III) indicated that these substrates were readily metabolized

TABLE III

Distribution of substrate activity in *Micrococcus radiodurans* metabolizing C^{14}-labeled carbohydrate

Substrate	Amount, mg	Radio-activity, μc	Radiochemical recoveries, %		
			In CO_2	In medium	In cells
D-Glucose-U-C^{14}	5	0.46	35	34	40
D-Fructose-U-C^{14}	5	0.48	41	16*	43
K-D-Gluconate-1-C^{14} + D-glucose	0.65 + 4.5	0.517	65	13	23

*Due to difficulties encountered in radiochemical assay, this value was obtained by subtracting the total recoveries in the CO_2 and cells from 100%.

FIG. 4. Radiorespirometric pattern of interval and cumulative recoveries from *Micrococcus radiodurans* metabolizing labeled yeast hydrolyzate and specifically labelled glutamic acid.

×——— DL-Glutamic acid-1-C^{14} (with DL-glutamic acid carrier).
●—— —— DL-Glutamic acid-1-C^{14} (with L-glutamic acid carrier).
△— — — DL-Glutamic acid-2-C^{14}.
○— — — C^{14}-labeled yeast hydrolyzate.

FIG. 5. Radiorespirometric pattern of interval and cumulative radiochemical recoveries from *Micrococcus radiodurans* metabolizing uniformly labeled glucose and fructose, and specifically labeled gluconate.

○——— D-Glucose-U-C^{14}.
×—— —— D-Fructose-U-C^{14}.
▼— — — Potassium gluconate-1-C^{14}.

R.T.U. = Relative time unit, which is defined as the time required for consumption of all the intact labeled substrate originally added (5).

by this organism for respiratory as well as biosynthetic functions. It is interesting to note that, in agreement with the manometric studies, fructose appears to be utilized somewhat better than glucose in so far as the respiratory functions are concerned.

The prompt conversion of C-1 of gluconate to CO_2 may indicate that a direct C_1–C_5 cleavage pathway is operative for the catabolism of glucose in this organism. It is also noteworthy that the presence of glucose in the incubation medium is essential for the utilization of gluconate.

Acknowledgment

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THE ISOLATION AND CULTURE OF THE NITRIFYING ORGANISMS

PART I. NITROBACTER[1]

G. W. GOULD[2] AND H. LEES[3]

Abstract

It has been found that two factors, accumulation of nitrate and inadequate aeration, seriously limit the growth of *Nitrobacter* under classical culture conditions. Techniques are described in which the effect of these factors is minimized and by which pure cultures of *Nitrobacter*, with cell densities at least 10 times the normal maximum, are easily and rapidly obtained.

Introduction

One of the problems that has always beset anyone investigating the biochemistry of the nitrifying organisms *Nitrosomonas* and *Nitrobacter* has been that of obtaining sufficient fresh cell material at one time for adequate study. Large scale aerated batch cultures (2, 4, 6, 11) provide a partial solution to the problem although they suffer from three disadvantages: the cell density of the fully grown culture is low, the cultures require constant attention, and the supply of cell material is spasmodic. We have now found that *Nitrobacter* can be grown with some facility either if means are taken to restrict the accumulation of nitrate in the culture or if the culture is intensively aerated; the best growth results from the simultaneous application of both these techniques. A preliminary note on this work has already appeared (7).

Materials and Methods

Growth medium for Nitrobacter.—This was essentially the medium G described by Lees and Simpson (11). In the present work, however, the medium was diluted 10 times with tap water before the sodium nitrite was added because it had been found (L. Herzenberg, private communication) that growth of *Nitrobacter* was more rapid in the more dilute medium. All growth experiments were carried out at 28° C.

Isolation of Nitrobacter.—An enrichment culture was first prepared from soil by several successive inoculations into 10-ml portions of dilute medium G (containing 70 μg nitrite-N/ml) in 50-ml flasks. A loop of the enrichment culture was then spread onto a matromycin–nitrite–agar plate (see below) and growth of the small, clear, *Nitrobacter* colonies was followed under the low-power (×100) microscope. When growth of the colonies was reasonably advanced, usually within 10 days of plating, single colonies were picked off

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with a wire loop and transferred to terramycin–nitrite–agar plates (see below) and growth again allowed to proceed. The *Nitrobacter* colonies appearing on this second set of plates were then seeded into the normal *Nitrobacter* growth medium. Nitrite oxidation in the seeded medium proceeded rapidly and the culture was apparently pure. Heterotrophic contaminants were tested for by transferring loops of the culture into or onto (i) nutrient agar, (ii) nutrient broth, (iii) peptone, 0.5%, plus glucose, 0.1%, in distilled water, (iv) dilute medium G with nitrite omitted but with ammonium sulphate, 0.05%, and peptone or glucose, 0.1%, added. The last two media were used in liquid form and also solidified with 1.5% Difco 'Noble' agar. Incubation in or on these media was carried out for at least 1 month and examination of the media for heterotrophic growth was performed both by eye and by examination under the microscope. No growth was detected. The purity of this culture has been checked at regular intervals both by ourselves and by Dr. C. T. Corke of the Ontario Agricultural College for almost two years and no contaminant has ever been found. The *Nitrobacter* cells isolated in this way are Gram-negative short rods, 0.5 $\mu \times 1.0$ μ. We have not, up to the present time, examined the organisms by electron microscopy and have therefore not observed the flagella described by Zavarzin and Legunkova (15).

Antibiotic–nitrite–agar plates.—A Seitz-filtered solution of terramycin or matromycin (5 ml at 400 μg antibiotic/ml) was pipetted into a sterile Petri dish. Melted nitrite agar (15 ml of dilute medium G containing 100 μg nitrite-N/ml plus 2% Difco 'Noble' agar) was poured at 50° into the dish and mixed with the antibiotic solution by swirling.

Bacterial reactors.—Two reactors (fermentors) were used. (1) A simple baffled reactor, capacity 5 liters, maximum impeller speed 240 r.p.m. (2) A baffled reactor with downdraught tube, capacity 500 ml, maximum impeller speed 1400. The base of this reactor was fitted with an outlet port connected to a porcelain bacterial filter candle of P 11 porosity (Baird & Tatlock, Ltd., Chadwell Heath, Essex, U.K.) through which culture fluid could be drawn off or fresh medium pumped in without loss of bacteria from the reactor and without contamination of the contents of the reactor. The reactors and contents were, of course, sterilized before use. Air pumped into the reactors was sterilized by passage through an autoclaved plug of asbestos wool lap (Cape Asbestos Co. Ltd., Park Street, London W.1., U.K.). Fresh medium pumped in through the candle was automatically sterilized in transit. The efficiency of the reactors in supplying oxygen to the cultures was assessed by measuring the SOX (Sulphite OXidation) values according to the method of Cooper *et al.* (5); these values are expressed as μmole O_2 absorbed/ml sulphite solution per hour. The maximum SOX value for the 5-liter reactor was found to be about 250 while that for the 500-ml reactor was about 1000. Both reactors were constructed by Mr. A. G. Silver of this Department.

Dry weights of *Nitrobacter* cells in a culture were determined either directly by weighing the washed dried cells from a culture sample or nephelometrically in an EEL nephelometer (Evans Electroselenium, Ltd., Welwyn Garden City,

Herts., U.K.) on the basis of a curve constructed from such dry weight determinations. This curve was regularly checked to confirm that it remained valid despite variations in growth conditions, age, and cell density of the cultures tested.

Nitrate was determined by the phenoldisulphonic acid method (14) and the yellow color developing was compared with that in standards and blanks by means of an EEL colorimeter fitted with a blue filter (maximum transmission at 430 mμ). Nitrite was determined by a Griess-Ilosva technique (14) and the red color developing was compared with that in standards and blanks in an EEL colorimeter fitted with a green filter (maximum transmission at 530 mμ).

The antibiotics terramycin and matromycin were a gift from Pfizer Laboratories, Folkestone, U.K.

Results

As the object of this work was to improve the growth of *Nitrobacter* it was first necessary to establish what growth rate and yield could be obtained from the organisms under 'normal' conditions. Sterilized batches of 700 ml of medium containing 100 μg nitrite-N/ml were therefore inoculated with a few drops of actively nitrifying *Nitrobacter* culture and aerated in 2-liter conical flasks by means of a stream of sterilized air fed through sintered glass spargers. When the initial 100 μg nitrite-N was almost completely oxidized, a further 200 μg nitrite-N/ml culture was added and so on. It was found that the addition of a nitrite increment greater than 300 μg nitrite-N/ml at one time resulted in a temporary depression of the oxidation rate; the addition of an increment greater than 600 μg nitrite-N/ml at one time prevented further oxidation for at least 1 week. When 2200 μg nitrite-N/ml had been oxidized, no further oxidation took place and cell growth ceased. The rate of nitrite oxidation was logarithmic until about 200 μg nitrite-N/ml had been oxidized; subsequently it was linear and at a rate partly dependent upon the rate of air flow through the culture. Enrichment of the air supply with CO_2 did not affect the rate of nitrite oxidation, but enrichment with pure O_2 caused a slight increase in the rate. The cultures showed a maximum oxidation rate of 7 μg nitrite-N oxidized/ml per hour with an air flow of 500 ml/minute per flask and finally attained a dry cell weight of 40 mg/liter of culture. The behavior of cultures to which various amounts of sodium nitrate were initially added together with the nitrite showed (Fig. 1) that an initial addition of 2000 μg nitrate-N/ml almost completely prevented nitrite oxidation whereas initial additions of between 100 and 500 μg nitrate-N/ml appeared to be very slightly stimulatory.

The first improvement in growth conditions was brought about by removing the nitrate accumulating in the cultures. An 18-in. loop of 0.5-in. diameter dialysis tubing was introduced into each 2-liter flask. After the addition of 700 ml medium the flask was sterilized, inoculated, and treated with 200 μg nitrite-N/ml increments until nitrite was being oxidized at the rate of 200 μg nitrite-N/ml per day. When this stage was reached, tap water was run through

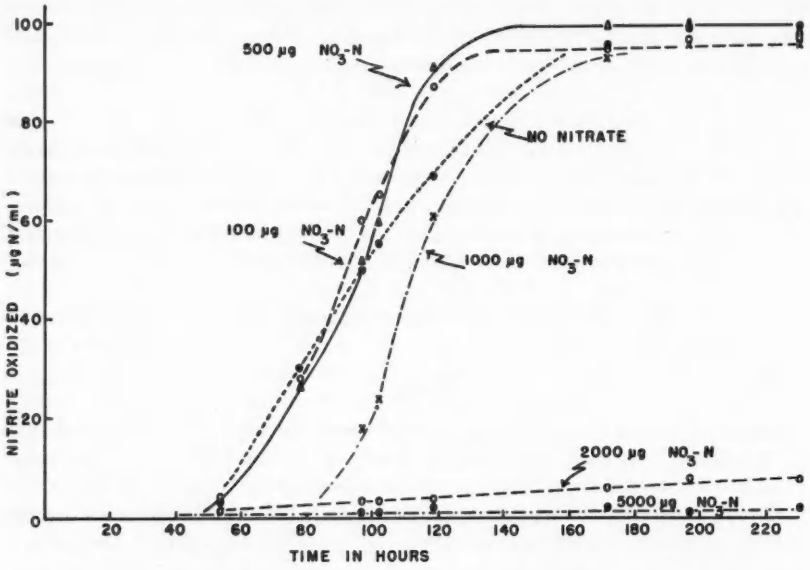

FIG. 1. The effect of initially added nitrate on nitrite oxidation by sparger-aerated cultures of *Nitrobacter* containing 100 µg nitrite-N/ml.

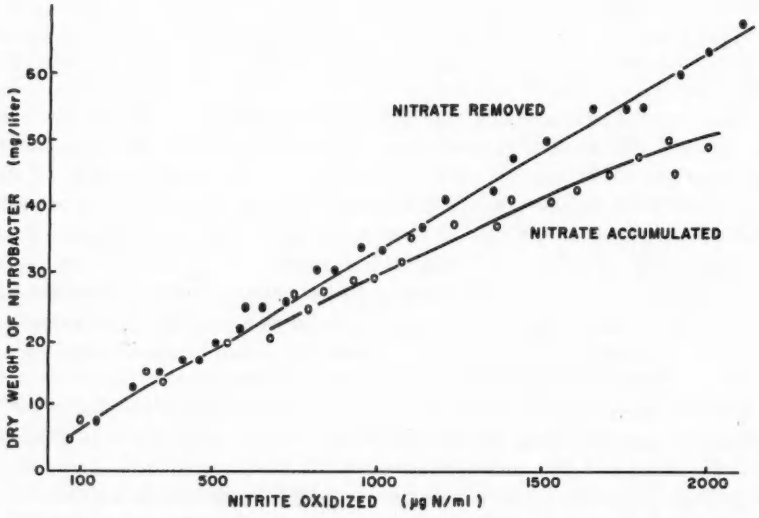

FIG. 2. The effect of periodically removing nitrate by dialysis from sparger-aerated *Nitrobacter* cultures.

the dialysis tubing for 2.5 hours during which time about half the nitrate present was removed by dialysis. A fresh 200 μg nitrite-N/ml was then added and the next day, when this had been oxidized, the dialysis was repeated and fresh nitrite added as before. A number of cultures were run in this way and no onset of any stationary phase was noted although 13,000 μg nitrite-N had been oxidized/ml culture. The dry weight of cells produced per liter of culture was slightly greater when nitrate was periodically removed in this way than when it was allowed to accumulate (Fig. 2).

The second improvement in growth conditions was brought about by increased aeration of the cultures. Cells grown in the 5-liter reactor, fed with air at 5 liters/minute, oxidized nitrite at rates up to 32 μg nitrite-N/hour at an impeller speed of 240 r.p.m. (SOX $\doteq$ 250) and showed a dry cell weight of 65 mg/liter when 2000 μg nitrite-N/ml had been oxidized.

A combination of nitrate removal and intense aeration was next carried out in the 500-ml reactor. The results of experiments designed to test the best impeller speed and the best rate of air supply showed that the higher the SOX value attained the more rapidly was the nitrite oxidized except in the early stages of growth of a culture. It was found that, until a culture had oxidized 100 μg nitrite-N/ml, high aeration was in fact inhibitory. The aeration rate in practice was limited by the fact that if the air flow were too rapid and the impeller speed too great, culture was lost as aerosol through the outlet port.

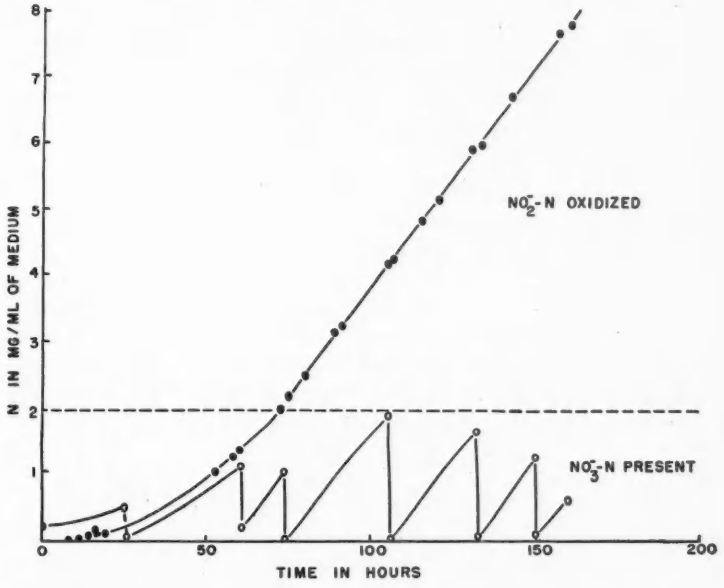

FIG. 3. The oxidation of nitrite by *Nitrobacter* cultures in the 500-ml reactor (SOX = 930). Nitrate was periodically removed by filtration.

The highest usable SOX value was 930, reached with an impeller speed of 800 r.p.m. and an air flow of 1 liter/minute. At this SOX value, growing cultures oxidized 100 μg nitrite-N/ml per hour. Nitrate removal was effected by periodically stopping the reactor and drawing off all the culture fluid through the porcelain filter candle and then forcing in fresh medium through the same candle. Sterile nitrite solution was supplied to the culture through a peristaltic pump fitted with an on–off timing device arranged to supply nitrite roughly at the speed at which it was being oxidized. The concentration of the nitrite solution was so adjusted that the additions did not appreciably alter the culture volume. The initial stages of nitrite oxidation under these conditions are illustrated in Fig. 3. The method of culture proved perfectly satisfactory and cultures oxidized 30,000 μg nitrite-N/ml and reached a dry cell weight of approximately 1.0 g/liter without any indication of the onset of a stationary phase.

Continuous Culture

Since continuous culture techniques have proved very useful in obtaining supplies of heterotrophic cells, the 5-liter reactor was adapted for the continuous culture of *Nitrobacter* by fitting it with a peristaltic pump connected to a sterilized reservoir of culture medium containing nitrite which, when the pump was adjusted to the correct speed, was fed into the reactor at the rate appropriate to steady-state continuous culture conditions. The grown culture of *Nitrobacter* was drawn off from the reactor by means of a siphon tube so that the culture volume remained constant at 5 liters. The behavior of an organism under conditions of continuous culture cannot be fully understood until three constants (8, 13) have been determined. They are (*a*) the yield constant, Y; (*b*) the saturation constant, K_s; (*c*) the growth constant, μ_m. The growth rate μ is some fraction, Y, of the substrate utilization rate; thus, where s is the substrate concentration and x is the cell concentration,

$$\mu = dx/dt = -Y \,.\, ds/dt$$

and over any finite period of growth

$$\frac{\text{weight of bacteria formed}}{\text{weight of substrate used}} = Y.$$

The constant K_s is the concentration of growth-promoting substance (e.g., nitrite in the case of *Nitrobacter*), present in limiting concentrations, at which μ is half the maximum growth rate (μ_m) attained when all growth-promoting substances are present in excess. Thus for a well-aerated *Nitrobacter* culture supplied with nitrite under conditions of continuous culture,

$$\mu = \mu_m \cdot \frac{\bar{s}}{\bar{s} + K_s}$$

where s is the residual concentration of nitrite in the steady state; i.e., the concentration of nitrite in the culture leaving the reactor when steady-state conditions have been established. It can be shown (8, 13) that under these conditions

$$\bar{s} = K_s \cdot \frac{D}{\mu_m - D}$$

where D is the dilution rate; i.e., the ratio of the rate of flow of medium to the volume of the culture.

Determinations based on the observed rate of nitrite oxidation in the 5-liter reactor by batch cultures of *Nitrobacter* (i.e., by cultures not grown under continuous culture conditions) showed that the lowest mean generation time at an SOX value of 250 was 12 hours. Therefore $\mu_m = 0.083$ hour^{-1}.

The yield constant was determined at an SOX value of 250 and a dilution rate of 0.03 hour^{-1}. This adjusted the growth rate of *Nitrobacter* to just less than half the maximum and ensured that the aeration was adequate during the experiment. A batch culture was first grown in the reactor until the 100 μg nitrite-N/ml initially added had been oxidized; continuous flow was then begun with medium containing this concentration of nitrite. The culture issuing from the reactor was regularly analyzed for nitrite and nitrate and, when these analyses showed that a steady state had been established (i.e., the issuing culture remained steady at 2.5 μg nitrite-N/ml and 97.5 nitrate-N/ml) dry cell weight determinations were carried out nephelometrically on the issuing culture. The level of nitrite in the ingoing medium was then raised, and the procedure repeated after a new steady state had been established. The dry cell weight concentrations were checked by direct estimation for every level of nitrite investigated above 250 μg N/ml. Below this point, the cell concentration was so low as to make direct checking of dubious value. Figure 4 shows

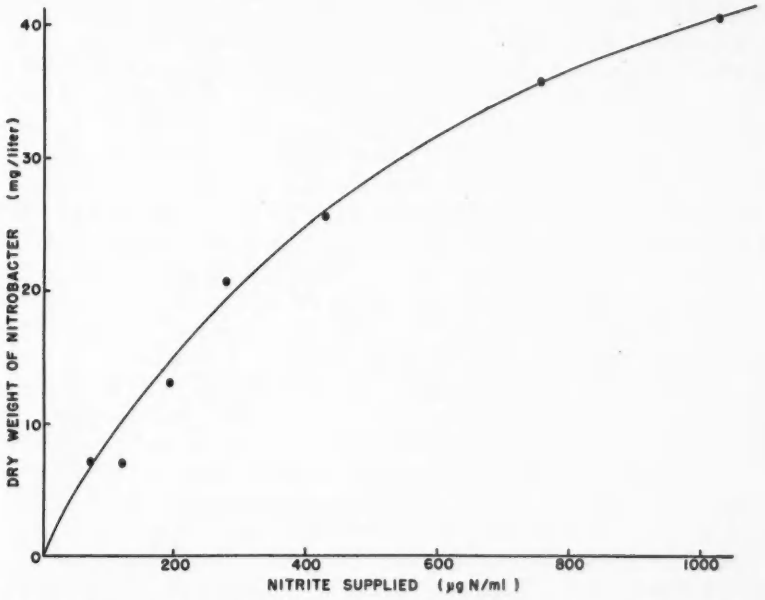

FIG. 4. The yields of *Nitrobacter* cells obtained under continuous culture conditions when nitrite was steadily supplied at the concentrations shown. Dilution rate = 0.03 hour^{-1} and SOX = 250.

the yield of *Nitrobacter* obtained, when a steady state had been established, and its relation to the concentration of nitrite in the supply medium.

Somewhat similar techniques were used to establish that the K_s for *Nitrobacter* is about 5 μg nitrite-N/ml (Table I).

TABLE I

Values of the saturation constant K_s for *Nitrobacter*

Nitrite concn. of added medium, μg N/ml	Dilution rate, hour^{-1}	Nitrite concn. in steady state ($\bar{s}$), μg N/ml	Mean nitrite concn. in steady state, μg N/ml	K_s nitrite, μg N/ml
1400	0.007	0.5, 0.3, 0.6, 0.6	0.5	5.7
1600	0.013	0.8, 0.6, 0.8, 1.2, 0.7, 0.7	0.8	4.3
1400	0.021	2.0, 1.5, 1.7, 1.4, 2.7, 2.5	2.0	5.9
1000	0.030	1.8, 2.9, 2.7, 2.3	2.4	4.3
1000	0.035	2.3, 4.2, 3.2, 3.3, 3.5	3.3	4.6
1000	0.042	4.1, 5.3, 6.0, 4.8, 4.7, 4.9	5.0	4.9
1000	0.044	3.0, 6.9, 4.8, 6.5, 8.0, 7.4	6.1	5.4

Discussion

The isolation and preservation of *Nitrobacter* in pure culture has never proved as difficult as the isolation and preservation of its ecological companion *Nitrosomonas*. Several workers (see ref. 15 for a detailed survey) have all obtained *Nitrobacter* in a pure, or relatively pure, state by a variety of techniques. Nevertheless, we feel that the antibiotic-plating technique we have used is worthy of some attention because it is simple, rapid, and (with suitable choice of antibiotics) possibly capable of eliminating every type of heterotrophic contaminant no matter how persistent such a contaminant may be.

The growth techniques described seem reasonably satisfactory. The technique of high aeration with periodic removal of nitrate yields a culture containing 1.0 g dry weight cells/liter in about two weeks. This is some 10 times the yield given by the usual sparger aerated culture over the same period of time. Under conditions of continuous culture, with a dilution rate of 0.03 hour^{-1} and nitrite supplied at 1000 μg nitrite-N/ml, a steady production of 40 mg dry weight cells/liter can be expected. If the culture volume is 5 liters, this means a steady production of 6 mg dry weight cells/hour or about 2 grams every 2 weeks. This method, of course, gives a considerably weaker cell suspension than does the high aeration batch culture technique with nitrate removal, but it has the advantage of supplying cells steadily and of demanding no attention save that involved in filling up the nitrite reservoir. The data in Fig. 4 and Table I should be of assistance to anyone proposing to culture *Nitrobacter* in this way.

It is of interest to note that Aleem (1) did not find nitrate to be as inhibitory to the growth of *Nitrobacter* as we have done. This may be due to some real physiological difference between his strain of organism and ours or it may be that, for some reason, our aeration techniques were more effective than his.

The latter suggestion is based on the finding (4) that nitrate inhibits nitrite oxidation by *Nitrobacter* more strongly as the oxygen tension of the gas phase is increased.

It is apparent from Fig. 4 that the yield of *Nitrobacter* cell material per amount of nitrite oxidized was not constant, so that Y, the yield constant, was not a true constant but decreased as the concentration of nitrate present rose. The free-energy data of Baas-Becking and Parks (3), and the assumption that *Nitrobacter* contains 50% carbon on a dry weight basis, can be used to show that the drop in free energy efficiency of the cells is from about 30% in the presence of 200–300 μg nitrate-N/ml to about 15% in the presence of 1000 μg nitrate-N/ml. These values are higher than the 7.8% found by Baas-Becking and Parks for old cultures of *Nitrobacter* containing much nitrate. This fall in free energy efficiency is of interest when one considers the parallel case in *Nitrosomonas* cultures (9) where a fall from about 40% to near 7% was indicated as the growth proceeded. A similar phenomenon has been observed with *Thiobacillus ferro-oxidans* (12) and may well be widespread (10) among chemolithotropic bacteria.

Acknowledgments

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ASPECTS OF DECOMPOSITION OF CELLULOSE IN CANADIAN SOILS

I. OBSERVATIONS WITH THE MICROSCOPE[1]

H. T. TRIBE[2]

Abstract

The course of cellulose decomposition in certain Canadian soils was followed by means of the microscope. Under the conditions of incubation provided, fungi initiated decomposition. Coarse mycelial forms, including a species of *Rhizoctonia*, were dominant. Species of *Humicola* and *Botryotrichum* and similar, sterile forms characterized by their "rooting branches" in cellulose film were subdominant. Following fungal growth, bacteria appeared and nematodes developed on both fungi and bacteria. The later stages of decomposition and disintegration of the cellulose were caused by larger fauna, including mites, collembolans, and enchytraeid worms. Nonhomogenous distribution of the various organisms in the soil samples resulted in considerable variation in rate and extent of decomposition of individual pieces of cellulose. Some pieces were entirely disintegrated, others were untouched after 5 weeks.

Introduction

Two experimental techniques were used in an investigation of the process of cellulose decomposition in soil: (*a*) direct microscopy of the cellulose and (*b*) estimation of the influence of the cellulose on mineral nitrogen availability in the soil and on carbon dioxide evolution. In this paper, results are reported of direct observations with the microscope of the succession of soil organisms colonizing cellulose from the time of burial until its disappearance. The form of cellulose used was cellulose film.[3] This is a pure regenerated cellulose and its great advantage is its transparency, which renders it a perfect substrate for microscopic observation. Some supplementary studies were made with filter paper, which being fibrous, may be expected to resemble more closely celluloses which occur naturally in soil than does cellulose film.

Material and Methods

Techniques

For observation of soil organisms on cellulose film, permanent stained microscope slides were prepared as follows: cellulose film was boiled in distilled water to remove plasticizers and then cut into pieces of approximately 0.5 × 1.0 cm. Whatman No. 1 filter paper was cut into similar pieces without pretreatment. Wet cellulose pieces were placed singly on 22 mm square cover slips, excess water drained off, and the cover slips buried vertically in pots of soil. After suitable intervals, cover slips were dug up and placed into picronigrosin in lactophenol (5) for several hours, then washed in lactophenol.

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Contribution No. 493 from the Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

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[3]British Cellophane Company Grade P.T. 300 Cellophane, kindly supplied by British Cellophane Company Ltd., Bath Road, Bridgwater, Somerset, England.

The back of each cover slip was then washed in distilled water, dried, and the cover slip mounted in lactophenol. The mount was sealed with several coatings of colorless nail varnish. In filter paper preparations it was sometimes necessary to tease the fibers apart before sealing, particularly when only slight decomposition had occurred. Small soil animals were removed and treated separately if it were desired to preserve them.

Certain fungi were isolated and cultured. Isolations from mycelium were made by cutting pieces of cellulose into small fragments and plating on Czapek–Dox medium containing 3% agar. To inhibit bacteria, 0.003% aureomycin was added as recommended by Dr. E. A. Peterson.[4] The development of fungal colonies was checked under the microscope to ensure that they arose from the desired mycelium.

Soils

The main study was made with a mull humus which possessed a good crumb structure. It was taken from the A_1 horizon of a brown forest soil under mixed deciduous trees at North Gower, Carleton County, Ontario.

Subsidiary studies were made with two samples of Granby sandy loam and one of Rideau clay. The former were taken from the surface of arable plots on the Central Experimental Farm in Ottawa. One, sample X, had received applications of farmyard manure over a long period of years while the other, sample N, had received neither manure nor fertilizers over the same period. The clay soil was taken from under grassland near Ottawa and possessed a good crumb structure. The pH of these soils was between 6.5 and 7.0. They were stored before use. Their mineral nitrogen content was determined as described by Tribe (7) and is recorded subsequently. Sieved samples (3 mm) of these soils were maintained in pots at laboratory temperatures. The mull humus was watered to 70% moisture holding capacity (m.h.c.) and allowed to dry to 40–50% m.h.c. before rewatering to 70%. The limits for the mineral soils were 60% and 30–40% m.h.c., respectively.

Results

Studies with Mull Humus

The course of decomposition followed the general pattern found in earlier studies with English soils (6). Primary colonizers were fungi, which were followed by bacteria. Nematodes developed in large numbers when the fungi were becoming moribund. Enchytraeid worms eventually devoured the partially decomposed cellulose film with its microbial colonizers, producing rather ill-defined excreta, mixed with much soil. The enchytraeids were seen in the soil at the beginning of the experiments but only attacked the film after several weeks, when it was extensively colonized by microorganisms.

The dominant fungus was a sterile mycelium, which was strongly cellulolytic and was identified as a species of *Rhizoctonia* (Figs. 1 and 2). The fungus was

[4]Personal communication.

TABLE I

Distribution of dominant fungi developing on cellulose film buried for various periods of time in a sample of mull humus

Period of burial (weeks)	Beaker number																				Totals		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	R	r	0
1	R	0	0	0	R	R	0	0	R	0	0	0	R	0	0	0	0	0	0	R	6	0	14
2	r	r	0	0	0	Rr	0	R	R	0	0	0	R	0	0	0	0	0	0	0	4	3	14
3	0	0	r	r	r	R	0	R	R	R	R	0	R	0	R	r	r	R	R	R	10	5	5
4	0	R	Rr	0	r	R	r0	R	r	r	R	R	R	0	R	0	R	R	R	pc	11	5	5
5	r	R	R	0	0	R	0	R	R	R	0	R	R	0	Rr	0	R	R	r	0	11	3	7
6	R	R	R	0	0	R	0	R	R	Rr	0	R	R	r	R	r	R	R	R	0	13	3	5
7	r	R	R	0	0	r	0	0	R	0	c	R	R	r	R	0	R	R	R	0	9	3	7
8	r	R	R	0	0	R	0	R	R	0	pc	R	R	0	R	0	R	0	R	0	10	1	8
																					74	23	65

NOTE: R = mycelium of *Rhizoctonia* sp.; r = "rooting" mycelium—*Humicola*, *Botryotrichum*, and allied species; pc, c = polycentric chytrids, chytrids; 0 = no significant colonization.

difficult to isolate and culture.[5] One group of fungi which occurred on cellulose film fairly abundantly was characterized by its manner of growth. These fungi grew over the surface and put characteristic "roots" into the thickness of the film (Figs. 3 and 4). These "roots" arose as small branches which grew vertically into the film for a short distance and then fanned out into a hyphal system parallel to the plane of the film. Sometimes (Fig. 3) but not always (Fig. 4), a clearly visible zone of solution of cellulose surrounded the "roots", an indication that penetration and growth of the "roots" into the film was effected by enzymic action. These fungi were less destructive than *Rhizoctonia*. Sometimes surface mycelium and "roots" bore numerous chlamydospore-like structures. Species of *Humicola* and the closely related genus *Botryotrichum* were isolated from the "roots." Sterile forms previously found in English soils were also seen; possibly these were imperfect forms of *Chaetomium* (6). In this paper these species are collectively termed "rooting fungi."

Another frequently occurring fungus was a polycentric chytrid (Fig. 5), often associated with the other fungi but only rarely dominant. It had a delicate growth habit and even when dominant did little apparent damage. It was present as a "rhizomycelium" as described for species of *Nowakowskiella* by Roberts (4) and was not seen to produce reproductive organs. Other chytrids were rare. In later stages of decomposition when nematodes had appeared, nematode-trapping fungi were sometimes found.

An estimate of the importance of the main fungi found on cellulose film buried under these conditions was made in the following experiment. A sieved sample of soil containing 95 p.p.m. nitrate nitrogen was thoroughly mixed and distributed among 20 beakers. Twelve cover slips, each bearing a piece of cellulose film, were buried in each beaker. Each week, for 8 weeks, a cover slip was taken from each beaker and examined microscopically; the results are shown in Table I. Despite the careful mixing of the soil there was extreme variation in the colonization and consequent fate of individual pieces

[5]The vigorous hyphae present in the film often autolyzed completely when the film was transferred to an agar plate. Those which survived made a slow and limited growth on Czapek–Dox agar and died out unless subcultured. Attempts were made to transfer them to potato dextrose agar, potato dextrose plus yeast extract, soil extract agar, and Czapek–Dox agar. Only on the soil extract agar did they develop to any appreciable extent. After maintenance on this medium for several weeks an isolate was brought back to Cambridge where Dr. J. Warcup succeeded in transferring it to Czapek–Dox agar containing yeast extract, on which it grew well. He confirmed that the isolate could be placed in the genus *Rhizoctonia* and that it was not *R. solani* Kühn.

FIG. 1. Cellulose film, buried 1 week in mull humus. Hyphae of *Rhizoctonia* sp. crossing a cover glass from the right. On reaching the edge of the film the hyphae branch out over it. ×60

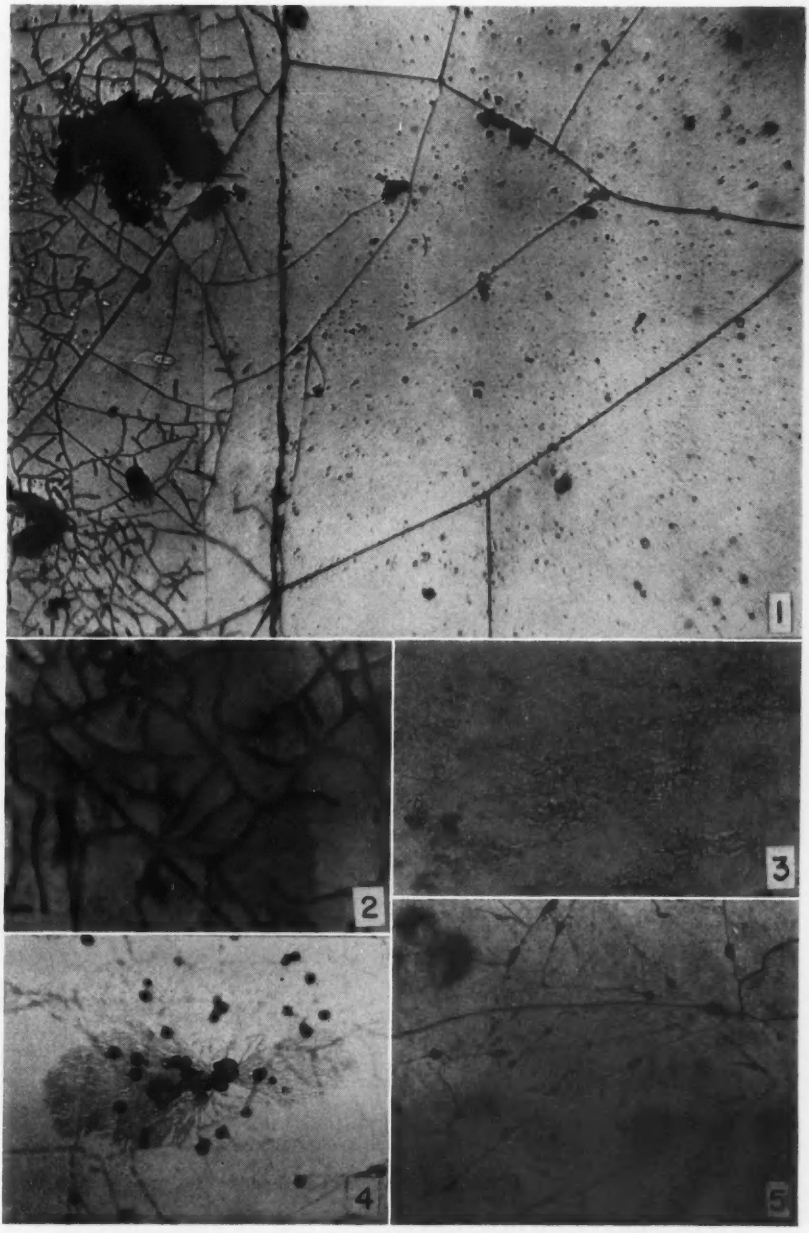
FIG. 2. Cellulose film, buried 3 weeks in mull humus. Typical *Rhizoctonia* hyphae growing over film. ×150

FIG. 3. Cellulose film, buried 3 weeks in mull humus. "Rooting branches" of a species of *Humicola* or allied genus. Note zones of solution of cellulose around the branches within the film and superficial hyphae above the focus. ×150

FIG. 4. A species of *Humicola* isolated from mull humus and grown over cellulose film placed on agar (Czapek–Dox without sucrose). The "rooting branch" in the film is bearing chlamydospores, as do superficial hyphae above the focus. ×150

FIG. 5. Cellulose film buried 4 weeks in mull humus. Rhizomycelium of a polycentric chytrid. Note pockets of enzymic erosion resulting from very fine branches penetrating the film. ×150

PLATE I



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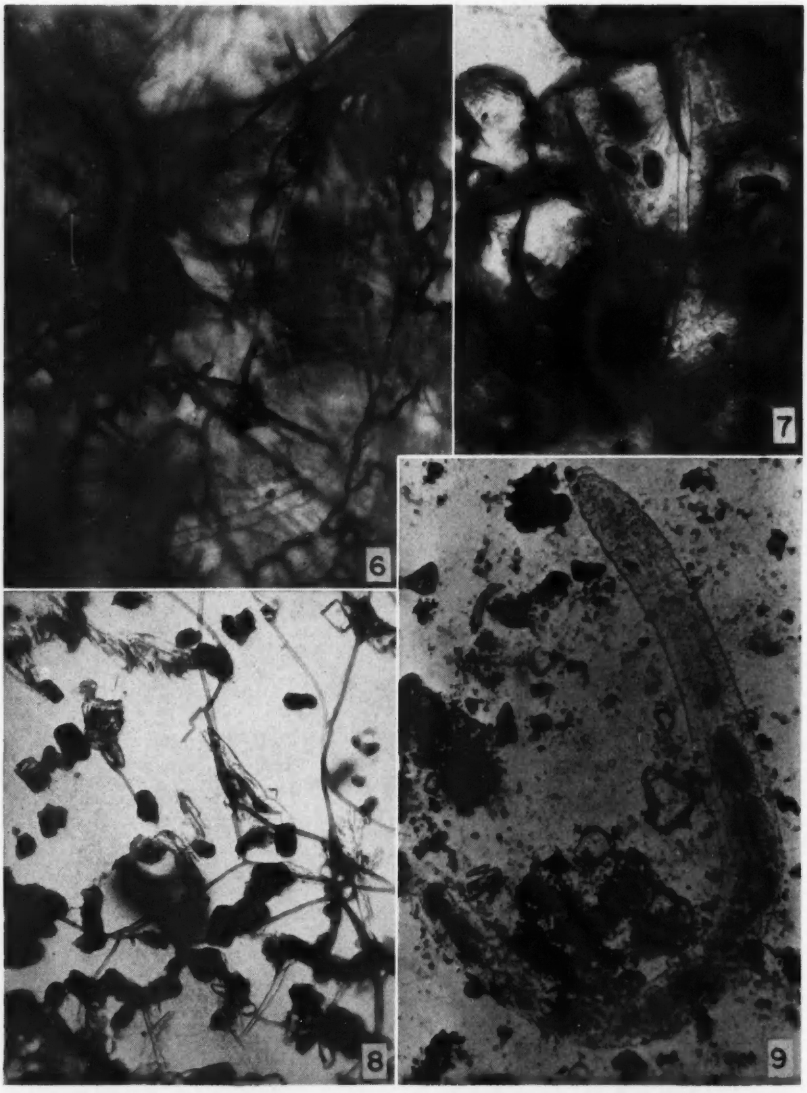

FIG. 6. Filter paper buried 4 weeks in soil N. *Rhizoctonia* hyphae investing the fibers. ×150

FIG. 7. Filter paper buried 6 weeks in soil X. Nematodes and their eggs amongst extensively decomposed fibers. ×150

FIG. 8. Filter paper buried 22 weeks in the clay soil. Residue consists of a few fibers, resistant hyphae of *Rhizoctonia*-like fungus, and droppings, probably from mites. ×150

FIG. 9. Enchytraeid worm from cellulose film in soil N. From a color transparency of a mount stained in chlor-zinc iodine in which some of the gut content stained blue, indicating the passage of undecomposed cellulose film through the gut. ×60

of film. For instance, in beaker No. 13, *Rhizoctonia* was observed on each piece of film withdrawn but from several beakers none was observed while in yet others its occurrence was sporadic. *Rhizoctonia* was able to travel through the soil by hyphal growth (Fig. 1) but in this experiment spread was limited, possibly because of the low concentration of film present and by the separation of one piece from another by the supporting cover slips. It appears therefore that propagules of *Rhizoctonia* were not abundant in the soil. They were probably pieces of resistant hyphae (8).

Physical disintegration of the film residue could result from its withdrawal from the soil after extensive decomposition by *Rhizoctonia*, especially if the soil were rather wet. In undisturbed soil, physical disintegration of residual film was caused by enchytraeid worms. Worms of sufficient length deposited their excreta into the soil and left no residue on the cover slip.

There was little disintegration in the mull humus soil until after 4 weeks, although by 6 to 8 weeks half of the pieces were partially or entirely eaten off. The extent of physical disintegration after different times of burial is shown in the first part of Table II.

TABLE II

Physical disintegration of cellulose pieces buried in certain Canadian soils for various periods of time

Soil	Period of burial (weeks)	Cellulose film disintegration[a]			Filter paper disintegration[a]		
		Heavy	Medium	Slight	Heavy	Medium	Slight
Mull humus	1, 2, 3	0	0	100			
	4	0	10	90			
	5	5	30	65			
	6	30	20	50			
	7	10	35	55			
	8	10	40	50			
Sandy loam (sample X)	7	55	45	0	40	45	15
Sandy loam (sample N)	7	25	40	35	40	15	45
Clay	9*	10	60	30	0	10	90
	22	60	30	10	80	15	5

[a]Heavy disintegration: piece mostly eaten; medium disintegration: piece partly eaten (approx. 25–75%); slight disintegration: piece entire or nearly so. Expressed as percentage of pieces examined. Percentages based on study of 20 pieces, except where marked with an asterisk, when based on 10 pieces.

Studies with Sandy Loam Soils

Preliminary experiments only were undertaken with these soils. Cover slips bearing pieces of cellulose film and others bearing filter paper were buried. Slides were made from five film and five paper pieces each week for 6 weeks; and after 7 weeks' burial a further 20 pieces of each form of cellulose were examined from each soil sample. Samples of soils X and N contained 22 p.p.m. nitrate nitrogen at the beginning of the experiment.

The general course of decomposition was similar to that in the mull humus, except that fungal colonization began later, rarely developing to any extent before 4 weeks' burial. Little difference was seen in the rate of decomposition between the two soil samples. Filter paper pieces were generally more resistant than the film, probably owing to their fibrous nature and because of their greater thickness. Nematodes were abundant (Fig. 7); of the larger fauna, enchytraeids (Fig. 9), mites, and collembolans were found. Mites were active in the soil from the beginning of the experiment, since pellets of excreta were often deposited upon pieces of film which were quite undamaged. That they could also develop on the cellulose was shown by the development of a young colony of over one hundred mites on one piece of filter paper.

The dominant fungus on both forms of cellulose was a *Rhizoctonia* which resembled very closely that from the mull; similar difficulties attended its isolation (Fig. 6). In soil X another fungus was found codominant with the *Rhizoctonia*, differing in growth form, in the production of granules near the hyphae, and in the longer persistence of its hyphae. This fungus could not be isolated. Fungi producing "roots" in the film occurred and isolates were obtained of species of *Humicola* and *Botryotrichum*. As in the mull, these were not very destructive. They were less evident in the filter paper, where they probably occurred in the lumina of the fibers, as is characteristic of growth of *Humicola* and similar fungi in cotton fibers (9). Hyphae were often seen in the filter paper fibers but no isolations were made from these to determine their identity. A few monocentric chytrids were found. *Stachybotrys atra* occurred only sporadically on both forms of cellulose, but was then very destructive. Species of *Cephalosporium* and *Sporotrichum*, sporing vigorously, were occasionally seen in soil X after 6 weeks. Their delicate hyphae did not appear to be destructive.

Variation in the degree of disintegration of the film and paper was again extreme (Table II). Some pieces were untouched after 7 weeks' burial whilst others were replaced by a mass of droppings or had disappeared altogether. All intermediate stages were seen.

Studies with Clay Soil

Cover slips were buried as in the sandy loam series but slides were made from film and paper pieces at intervals of 3, 9, and 22 weeks. The sample contained 31 p.p.m. nitrate nitrogen at the beginning of the experiment.

There was little colonization after 3 weeks. After 9, most of both film and filter paper were colonized by a coarse sterile mycelium resembling the *Rhizoctonia*-like fungus from soil X. The hyphae were strongly persistent. Occasional "rooting fungi" were present and a species of *Humicola* and one of *Botryotrichum* were isolated from film. One other fungus which appeared in quantity was a species of *Cephalosporium*, abundant on both forms of cellulose. It penetrated the cellulose with its delicate mycelium but was not destructive. The pieces of film were partially eaten and numerous droppings indicated that mites were responsible; the paper pieces were less eaten (Table II). After 22 weeks only droppings, hyphae of the dominant fungus, and a few residual fibers remained in place of the cellulose (Fig. 8).

Discussion

The results summarized in Table I demonstrated that the presence of certain fungi was necessary for initiating decomposition of cellulose film in the mull soil studied. Under the conditions of incubation provided, only the *Rhizoctonia* sp., the "rooting fungi", and chytrid fungi were primary colonizers and in their absence the cellulose was scarcely attacked. In the latter case a limited bacterial growth sometimes developed eventually; very occasionally a small piece of uncolonized film would be nibbled by some soil animal. Virtually no damage resulted from either of these actions. The combination which decomposed the cellulose most thoroughly was that of *Rhizoctonia* and enchytraeid worms. *Rhizoctonia* was able to reduce a piece of film to a mushy condition in 3 weeks, and the worms could disperse the residue within 5 weeks from the time of burial.

In these soils, mites, enchytraeids, and collembolans were seen to be active on cellulose colonized by microorganisms. In the absence of these animals, even extensively decomposed cellulose bearing moribund microorganisms remained *in situ* for long periods. One of the major effects of the activity of the soil fauna is comminution of organic materials. Only a part of the material which passes through the gut can be utilized by the animal, and the biochemical role of the soil fauna is at present extremely obscure (Murphy, in Kevan (2, p. 117)). The fate of both mite and enchytraeid droppings, which constitute a large fraction of some natural humus forms (Kubiena, in Kevan (2)), appears to be a very slow decomposition. Kühnelt (3, p. 124) states that decomposition of organic residues in the gut of oribatid mites is very extensive and almost no cellulose residue remains. In the present study, a few cover slips bearing excreta of mites and of enchytraeid worms were treated with chlor-zinc iodine, according to Artschwager's procedure (1), in order to detect the presence of any undecomposed cellulose film. The blue coloration, which indicated cellulose, was not found in the mite droppings but some of the enchytraeid excreta did contain cellulose. It is not yet possible to say whether such particles of cellulose in these droppings are readily decomposable or whether they remain intact for a long period.

With reference to the nature of the fungi in these soils, the coarse mycelial forms were dominant and were the most destructive, followed in both cases by the "rooting fungi". *Stachybotrys atra* was destructive but only occurred sporadically; the other species mentioned were of minor significance under the environmental conditions provided. Coarse mycelial fungi had been found in two samples of English soils in which studies had been made on buried cellulose film. Both were light loams and the occurrence of these fungi was patchy. One was a characteristic sterile mycelium, differing from any of the forms mentioned in this paper, and the other had been identified, though without pathogenicity tests, as *Rhizoctonia solani* Kühn (Tribe, unpublished data). When they did occur they were destructive and dominant; in their absence "rooting fungi" were dominant.

Although pure cellulose is almost certainly decomposed by a smaller variety of organisms than are most natural residues, any organic substrate is presumably subject to the action of a similar association of soil organisms, leading to decomposition and dispersal. In the mull humus studied here, the absence of one fungus, the *Rhizoctonia* species, greatly reduced the rate and extent of initial decomposition and consequently of later dispersal and continued decomposition. The presence of specific fungi may be found also to have an important bearing on the extent and rate of decomposition of the more complex natural residues.

Acknowledgments

It is a pleasure to record my thanks to Dr. H. Katznelson, Director, Microbiology Research Institute, Canada Department of Agriculture, Ottawa, for generous provision of facilities for this work and to him, Dr. A. G. Lochhead, and Dr. I. L. Stevenson for their criticism of the manuscript of this paper

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ASPECTS OF DECOMPOSITION OF CELLULOSE IN CANADIAN SOILS

II. NITRATE NITROGEN LEVELS AND CARBON DIOXIDE EVOLUTION[1]

H. T. TRIBE[2]

Abstract

The effect of addition of cellulose film on the level of nitrate in a mull humus soil was studied over a period of 16 weeks. During the early stages of decomposition, nitrate was taken up from the soil, leaving it deficient in nitrate for up to 7 weeks. In later stages of decomposition, some of this nitrate was released again. Carbon dioxide was produced from the cellulose film over the whole experimental period. The results were broadly correlated with previous observations on the succession of microorganisms and fauna on cellulose film. The role of the fauna in nitrogen mineralization is discussed.

Introduction

This paper reports the second part of an investigation into the process of cellulose decomposition in soil. In the previous paper (8) direct microscopic observations of the nature of the microbial population concerned in cellulose decomposition in certain Canadian soils were described. In the present study the influence of cellulose on the availability of mineral nitrogen in one of these soils was determined. The effect of cellulose on carbon dioxide production was also followed over the same period of time. It is well known that addition of organic material with a wide carbon–nitrogen ratio to soil causes a depletion of available nitrogen in that soil, since the nitrogen is used for the synthesis of protoplasm by the microorganisms which develop on the material. Cellulose film,[3] which was used in the major part of the previous study, was also used here. It is a pure regenerated cellulose and when washed free of plasticizers contains only 0.05% nitrogen (by Kjeldahl estimation). Virtually all the nitrogen required by the population developing on it must therefore be derived from the soil.

Material and Methods

A leaching tube technique, in principle similar to that of Stanford and Hanway (7), was employed for extraction of mineral nitrogen from the soil samples. Fifty-gram soil samples containing approximately 50% moisture (= 57% moisture holding capacity) were sieved through 3 mm mesh and were placed in the leaching tubes. These were prepared from 10-in. lengths of 25

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[2]School of Agriculture, University of Cambridge, England; National Research Council of Canada Postdoctorate Fellow 1957–1958 in the Microbiology Research Institute.
[3]British Cellophane Company Grade P.T. 300 Cellophane, kindly supplied by British Cellophane Company Ltd., Bridgwater, Somerset, England.

mm diameter glass tubing and closed at the lower end by 1-hole stoppers fitted with a short length of 6 mm diameter glass tubing. Plastic gauze prevented soil dropping through this drainage tube. Mineral nitrogen was extracted by leaching the tubes with 60 ml distilled water, added in three portions of 20 ml. The volume of leachate which came through was brought to 60 ml.

Mineral nitrogen was determined by means of a modification of Mason's method (5). The principle of this method is the reduction of nitrate to ammonia by ferrous sulphate in the presence of silver ions as catalyst and distillation of the ammonia into boric acid. The procedure determines nitrate, nitrite, and ammonia together. Mason's method was adapted for use in Markham's steam distillation apparatus (4) as follows: into 10 ml leachate was dissolved 0.4 g analytical grade hydrated ferrous sulphate crystals and the solution transferred to the Markham still; 5 ml 0.8% silver sulphate solution were then run in. Two milliliters 50% w/v sodium hydroxide was added and the mixture steam-distilled for 3 minutes. Ammonia was collected in 10 ml 0.5% boric acid containing mixed indicator (Conway and O'Malley (1)) and titrated against 0.01 *N* hydrochloric acid.

Ammonia in the leachate was determined directly by steam distillation without added ferrous or silver sulphates. Nitrite was tested for by the method of Woodward (9).

Carbon dioxide was collected from leaching tubes fitted with 2-hole stoppers and connected to a supply of CO_2-free air. The air flow was adjusted by screw clips to permit air to pass slowly over the soil and bubble through Pettenkoffer tubes containing 25 ml carbonate-free 0.1 *N* sodium hydroxide. At suitable intervals the soda was removed, treated with excess 10% barium chloride to precipitate carbonate, and titrated against 0.1 *N* hydrochloric acid, using a phenolphthalein–thymolphthalein mixed indicator.

The sample of mull humus used was the same as that employed for the microscope observations on buried cellulose film described in the previous paper (8). It was obtained from the A1 horizon of a brown forest soil under mixed deciduous trees at North Gower, Carleton County, Ontario.

Cellulose film was washed free of plasticizers by boiling in distilled water, air-dried, and then cut into pieces of approximately 1.0×0.5 cm.

The effect of added cellulose film on soil nitrate was studied by a repeated leaching procedure (expt. 1, below). The distilled water leachings were made at intervals on the same soil samples and the soil moisture adjusted after each leaching to approximately 70% of its moisture holding capacity (m.h.c.) by applying a vacuum line to the drainage tube. In a confirmatory experiment (expt. 2), tubes were leached once only. Indicator slips bearing pieces of cellulose film were included in these tubes, and were examined microscopically after the leaching. Replicate tubes fitted for collection of carbon dioxide were set up with each series. It was found that the ammonia concentration in these leachates never exceeded 1 p.p.m. and that nitrite was absent. The analyses of total mineral nitrogen therefore represented nitrate nitrogen.

Results

Experiment 1

Six tubes were set up; two contained 0.5% of cellulose film pieces, two tubes contained 0.25% film, and two were without film. Leachings were made at weekly intervals for 10 weeks and then at 2-week intervals until 16 weeks. The carbon dioxide evolution from six similar tubes was determined at twice-weekly intervals up to 7 weeks and thereafter as shown in Fig. 2. All the tubes in this experiment were incubated at laboratory temperature for the first 4 days under nearly saturated conditions, when the soil samples were adjusted to approximately 70% m.h.c. It may therefore be assumed that the nitrate content of the samples at 4 days was near to zero.

Figure 1 illustrates release of nitrate expressed as cumulative determinations of nitrate nitrogen. The control soil samples released nitrate from the beginning of the experiment and the rate began to fall off toward the latter half of the 16-week period. Where 0.25% of cellulose film pieces were added, no nitrate was released until the fifth week and thereafter it was released at an

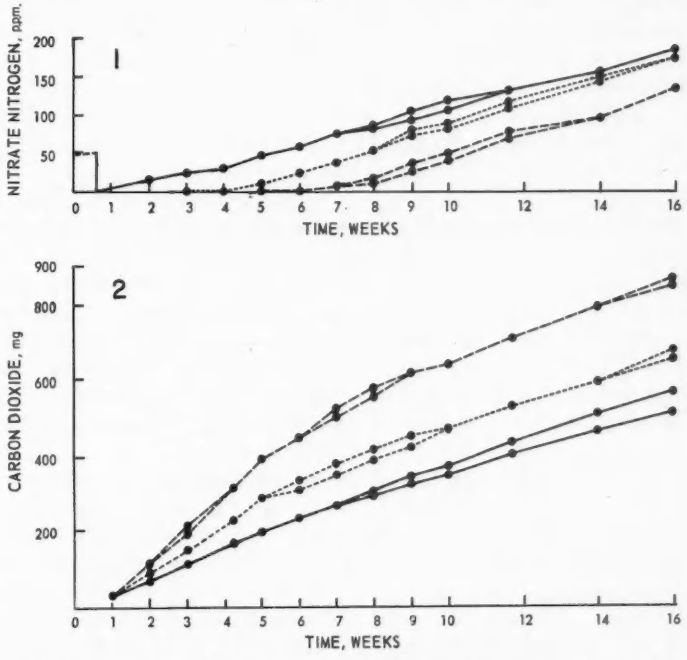

FIG. 1. Nitrate release from mull humus incubated with and without added cellulose film. Points represent cumulative determinations. Nitrate from control soil, solid lines; from soil plus 0.25% cellulose film, short dashes; from soil plus 0.5% cellulose film, long dashes.

FIG. 2. Carbon dioxide evolution from mull humus incubated with and without added cellulose film. Points represent cumulative determinations. Carbon dioxide from control soil, solid lines; from soil plus 0.25% cellulose film, short dashes; from soil plus 0.5% cellulose film, long dashes.

almost constant rate. Where 0.5% was added, 7 weeks elapsed before nitrate was detectable and then nitrate release ran parallel to that recorded for the 0.25% addition. The analyses of the duplicate series agreed closely. The nitrate present soon after its release from the soil containing pieces of film was subtracted from that accumulated in the control soil by that time, which gave an estimate of the nitrate nitrogen taken up by microorganisms decomposing the film. In 5 weeks 37 p.p.m. nitrate nitrogen were required for decomposition of 0.25% pieces and after 7 weeks 70 p.p.m. had been taken up for 0.5% pieces. The differential between the nitrate release graphs following addition of the two concentrations of cellulose film pieces remained almost constant up to 16 weeks, when it was 41 p.p.m.

Thus 35 to 41 p.p.m. were required for decomposition of 0.25% film under the experimental conditions and since 1 p.p.m. is 0.05 mg in 50 g soil, this represents 1.75 to 2.05 mg weight of nitrate nitrogen. After 16 weeks, nitrate release following addition of 0.25% film had almost reached that released by the control soil, indicating that the nitrogen taken up in the first 5 weeks was mostly released in the following 11 weeks.

Carbon dioxide evolution is shown in Fig. 2 as cumulative determinations. When nitrate uptake had ceased, by 5 weeks in the tubes containing 0.25% film, 77 mg carbon dioxide in excess of the controls were produced. By 7 weeks, 240 mg additional carbon dioxide were evolved from the tubes containing 0.5% film. After 16 weeks the average carbon dioxide evolution was 115 mg and 308 mg, respectively. The figures varied between 74 and 155 mg and 272 and 344 mg, which wide variation was partly caused by progressive divergence of the control duplicates. Estimates of the percentage decomposition of the cellulose pieces may be made from these figures but reflect the above variations. At 0.25% film concentration 50 g soil contained 125 mg film, the dry weight of which was 112 mg. Assuming the film to contain 45% carbon, 50 mg carbon was added to the soil and hence would theoretically yield 183 mg carbon dioxide. After 5 weeks, therefore, $77/183 = 42\%$ of the film was evolved as carbon dioxide and 40 to 80% by 16 weeks. Similarly, in 0.5% concentration, $240/366 = 65\%$ was decomposed by 7 weeks and 74 to 94% by 16 weeks.

Of the 115 mg total carbon dioxide collected from 0.25% film, 77 mg or 67% was evolved by the time that nitrate uptake had ceased. The corresponding figure for 0.5% film was 78%. Decomposition of the cellulose had therefore continued after the nitrate requirements of the microorganisms responsible had been satisfied.

Experiment 2

The tubes in this experiment were leached once only. Eight tubes were set up, each containing 0.5% cellulose film pieces and eight indicator cover slips bearing cellulose film. After 2, 3, 4, and 7 weeks, duplicate tubes were leached and slides made from the indicator slips. Two control tubes (without cellulose film) were leached for nitrate after 7 weeks' incubation. Carbon dioxide evolution was estimated at weekly intervals from three tubes containing

0.5% pieces of film and three controls. None of the tubes was leached at the beginning of the experiment and the initial nitrate nitrogen content of the soil was 46 p.p.m.

The results in Figs. 3 and 4 show that the trends were as before. In these soil samples sufficient nitrate was present at the beginning to prevent the nitrate becoming exhausted. Nitrate requirement by the film under these conditions was similar to that found in the repeatedly leached series. The greatest difference from the control was at 4 weeks and indicated an uptake of 66 p.p.m., or 3.3 mg in 50 g soil. The comparable figures with those in experiment 1, at 7 weeks, were 55 p.p.m. (2.75 mg).

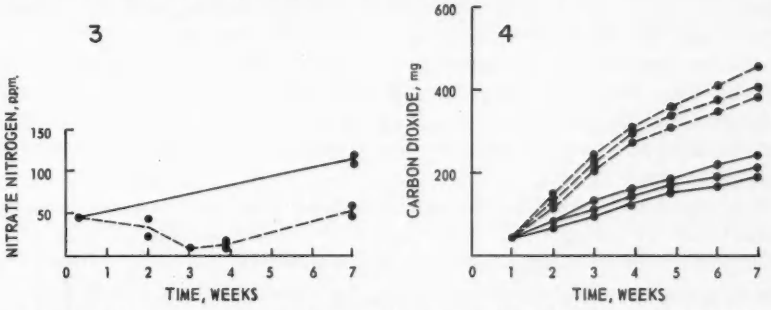

FIG. 3. Nitrate release from mull humus incubated with and without added cellulose film. Points represent duplicates, leached once only. Nitrate from control soil, solid lines; from soil plus 0.5% cellulose film, long dashes.

FIG. 4. Carbon dioxide evolution from mull humus incubated with and without added cellulose film. Points represent cumulative determinations. Carbon dioxide from control soil, solid lines; from soil plus 0.5% cellulose film, long dashes.

After 2 weeks the indicator slips from the duplicate yielding 46 p.p.m. nitrate nitrogen were much less decomposed than that which yielded 22 p.p.m. At and after 3 weeks the pieces were extensively decomposed. Decomposition was much more uniform than in the experiments recorded in the previous paper (8) where only 12 pieces of film, mounted on cover slips, were put into 500-ml beakers of soil. This may have been because of a more uniform moisture content in the tubes but more probably because of the closer packing of the pieces of film, which was such that the dominant fungus, *Rhizoctonia* sp., was rapidly able to spread from one piece to the next.

At 4 weeks when the nitrogen difference was greatest, 110 to 165 mg carbon dioxide had been evolved in excess over the controls; this represented 30 to 45% decomposition. By 7 weeks 162 to 256 mg excess carbon dioxide were produced, indicating 44 to 70% decomposition. These figures and the variation were in broad agreement with those of the previous experiment.

Discussion

During the early stages of decomposition of cellulose film nitrate nitrogen was taken up from the soil and where nitrate was in limited initial supply the soil became totally deficient in nitrate during the period of uptake. Thereafter nitrate reappeared in the leachates from the soils treated with cellulose

and the rate of release from these was greater than from the controls. This indicated that some of the nitrate initially taken up was subsequently released and from the soil containing the lower concentration of film almost all of it was released in 11 weeks (Fig. 2). Carbon dioxide was evolved from the film over the whole 16-week period. Most of this was produced by the time nitrate began to be released again by the soils containing cellulose.

This movement of nitrate and evolution of carbon dioxide may be broadly correlated with the biological observations described in the previous paper (8), as follows: Nitrate nitrogen was first taken up by fungi and bacteria which developed on the cellulose. When they reached maximal development the requirement was reduced, or ceased, and nitrogen began to be released into the soil. It is likely that two processes contributed to this release: autolysis of microorganisms and nitrogenous excretion by the soil microfauna which was by then becoming active on the cellulose. Carbon dioxide evolution resulted from the activities of fungi, bacteria, and animals and would therefore be expected to continue after the nitrogen requirements of the microorganisms had been met.

Nitrogenous excretion by the soil microfauna has not been considered of importance in nitrogen transformations by microbiologists, though it is characteristic of the animal body to excrete nitrogen in a fairly simple form. Nothing seems to be known concerning the nature of the nitrogen excreted by free-living nematodes. Mites excrete guanine in the form of solid particles mixed with their droppings (Hughes (3)). This would presumably be rapidly decomposed to ammonia by soil bacteria. Earthworms are also said primarily to excrete guanine, which is decomposed internally by phagocytic coelomic corpuscles and its decomposition products pass out "in a continual stream through the nephridia" (2). According to Needham (6), the earthworms *Eisenia foetida* and *Lumbricus terrestris* both excrete up to half of their nitrogen as protein, probably mucus, and a high percentage of the remainder as urea and ammonia. It is highly probable that enchytraeid worms, being small annelid worms, have a similar physiology. Earthworms were probably excluded from these experimental soil samples by reason of soil storage and the sieving treatment. There is evidence therefore that the activity of soil animals leads to mineralization of nitrogen.

With reference to the two procedures used in experiments 1 and 2, the repeated leaching method has certain advantages over the use of replicates. Firstly, by making serial leachings on the one sample, variation between the replicates is eliminated. For the same reason it would be desirable to make the nitrate extractions and carbon dioxide collection from the same tubes. A second advantage is that few tubes need to be set up, which is useful in preliminary trials. A disadvantage of the procedure is that if distilled water be used as leaching agent, the nitrate content is reduced to zero after each leaching. With the sample of mull humus used in these experiments the rate of nitrate release (initially approximately 12 p.p.m. per week) was adequate for the decomposition process but where a slower rate of release may be

expected, decomposition may be limited thereby. For the maintenance of nitrate level and also for the recovery of indicator slips during intermediate stages of decomposition, use of a number of replicates is considered necessary. The complementary use of both procedures is therefore to be recommended in investigations of this nature.

Acknowledgments

It is a pleasure to record my thanks to Dr. H. Katznelson, Director, Microbiology Research Institute, Canada Department of Agriculture, Ottawa, for generous provision of facilities for this work and to him, Dr. A. G. Lochhead, and Dr. I. L. Stevenson for their criticism of the manuscript of this paper.

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STUDIES ON A SOIL ACHROMOBACTER WHICH DEGRADES 2,4-DICHLOROPHENOXYACETIC ACID[1]

GORDON R. BELL[2]

Abstract

Cells of an *Achromobacter* sp. possess what appears to be an adaptive system that is capable of oxidizing 2,4-dichlorophenoxyacetic acid (2,4-D). The 2,4-D oxidizing system was most active at 30° C. at pH 6.0 or lower, and in barbital buffer. The organism can oxidize a wide variety of 2,4-D analogues, and 2,4-dichlorophenol without prior adaptation, making analysis of mechanisms difficult. For rates of oxidation comparable with that of 2,4-D the organism appeared to have the following structural requirements: a free ortho position and, more important, an unchlorinated ortho position; a free carboxyl group on the side chain preferably β to the ethereal linkage; a chlorine atom in the para position and no more than two chlorine atoms in the ring whether or not an ortho position is free. Proposed and established pathways for the degradation of 2,4-D are presented and a possible new pathway involving 2,4-dichloroanisole is suggested.

Introduction

It is now well established that microorganisms are largely responsible for the detoxication of hormone herbicides in soil, and several species of bacteria able to decompose certain chlorophenoxyacetic acids in soil and synthetic media have been isolated and studied. Audus (1, 2) isolated an organism designated *Bacterium globiforme* which detoxicated 2,4-dichlorophenoxyacetic acid (2,4-D) but sparse growth made metabolic studies difficult. Audus and Symonds (3) presented evidence showing the accumulation of at least two unidentified, phytotoxic intermediates in culture media. Jensen and Petersen (11) isolated a strain of *Flavobacterium aquatile* and a *Corynebacterium* sp. able to decompose 2,4-D and both 2,4-D and 2-methyl-4-chlorophenoxyacetic acid (MCPA) respectively. However, Weeks (25) showed the *F. aquatile* to be a strain of *Sporocytophaga congregata*. Evans and Smith (9) examined two Gram-negative bacteria one of which utilized 4-chlorophenoxyacetic acid (4-Cl-PAA) as the sole source of carbon and formed 2-hydroxy-4-chlorophenoxyacetic acid and 4-chlorocatechol; the other utilized 2,4-D and accumulated a compound tentatively identified as 2,4-dichloro-6-hydroxyphenoxyacetic acid (6-OH-2,4-D). Further work by Professor W. C. Evans in co-operation with Brown and McCall (7) showed that the metabolite of 2,4-D was not 6-OH-2,4-D nor was it 4-chloro-2-hydroxyphenoxyacetic acid or 2-chloro-4-hydroxyphenoxyacetic acid.

Following a series of experiments on the behavior of 2,4-D in soil, Stapp and Spicher (18) isolated a bacterium closely related to *F. aquatile* but differing in such important characteristics that they named it *Flavobacterium peregrinum*.

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Contribution No. 168 from the Pesticide Research Institute, London, Ontario. Some of this material was presented at the Seventh Annual Meeting of the Canadian Society of Microbiologists, London, Ontario.
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Their data indicated that the organism was able to cleave the aromatic moiety of 2,4-D. Subsequently, Spicher (15) showed an optimum pH range of 7.0–7.5 for 2,4-D decomposition by *F. peregrinum* and an optimum growth temperature of 25° C.

The characteristics of a new soil *Corynebacterium* somewhat resembling Audus's *B. globiforme* and the *Corynebacterium* of Jensen and Petersen (11) were described by Rogoff and Reid (14). The organism oxidized 2,4-D, 2,4-dichlorophenol (2,4-DCP), and possibly 2-phenoxyethanol and phenoxyacetic acid. The high percentage recovery of CO_2 and chlorine from metabolized 2,4-D and an R.Q. of 1.12 indicated that the molecule was completely dissimilated. Walker and Newman (24) isolated a *Mycoplana* sp. which utilized as growth substrate 2,4-D, 4-Cl-PAA, and 4-chlorophenol, but less readily MCPA. Growth and 2,4-D decomposition were stimulated by 0.05% yeast extract. They concluded that 2,4-D was completely degraded by resting cells and that these cells were able to oxidize 2,4-D, MCPA, 2,4-DCP, phenol, and a variety of other aryloxyacids.

Steenson and Walker (19) studied a strain of *F. peregrinum* n. sp. (Stapp and Spicher) and two *Achromobacter* species which utilized respectively 2,4-D, 4-Cl-PAA; 2,4-D, MCPA, and 4-Cl-PAA; and 4-Cl-PAA only. Chloride release and oxygen uptake values indicated that *F. peregrinum* n. sp. decomposed 2,4-D to C_2 or C_3 units containing chlorine. Subsequently, Steenson and Walker (20) gave evidence to suggest that 2,4-DCP and 4-chlorocatechol rather than 6-OH-2,4-D and 3,5-dichlorocatechol (3,5-DCC) were intermediates in the dissimilation of 2,4-D by young, 2,4-D-adapted *Achromobacter* cells. Thus, cleavage of the side chain of 2,4-D appeared to occur prior to oxidation of the ring. Bell (6) also described certain physiological characteristics of a new soil *Achromobacter* species which could utilize as principal source of carbon 2,4-D, MCPA, 4-Cl-PAA, and related compounds. Resting cells oxidized 2,4-D, MCPA, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2,4-DCP and released chloride quantitatively from 2,4-D. More recently Steenson and Walker (21) studied the adaptive patterns of *F. peregrinum* and *Achromobacter* sp. cells cultured on peptone media containing 2,4-D, MCPA, and other aromatic substrates.

The present paper gives a record of the outcome of attempts to clarify the mechanism of oxidation of 2,4-D (see Fig. 7) and to determine the degree of specificity and the characteristics and properties of the 2,4-D oxidizing system of the *Achromobacter* sp. previously described (6).

Materials and Methods

Reagents

Phenoxyacids (Eastman Kodak) used were of Reagent Grade. A sample of 2,4-D free of phenols was used in culture media but for manometric experiments it was recrystallized three times from hot chloroform to an uncorrected, constant melting range of 140–142° C (literature value (10), 140–141° C).

All other compounds were highly purified in order to reduce possible contaminants, such as the toxic pink substance found in some reagent grade 2-chlorophenoxyacetic acid, and their uncorrected melting points are given below.

3-(2,4-Dichlorophenoxy)propionic acid (3-(2,4-D)P), m.p. 93° C (5), was synthesized by condensing Na 2,4-DCP and 3-chloropropionic acid.

6-OH-2,4-D, m.p. 129–132° C (literature value (8) 132° C) was synthesized by R. W. White according to the method of Cavill and Ford (8).

3,5-DCC, m.p. 85–86° C (literature value (8) 85–86° C) was synthesized by R. W. White. Stock solutions were freshly prepared just before use.

Cells

The *Achromobacter* sp. was grown for 6 days at 28° C in the aerated mineral – yeast extract – L-malic acid medium (23YM) previously described (6). Cells were harvested in a Sharples centrifuge, washed four times with distilled water, and suspended in distilled water before use.

Manometry

Conventional Warburg respirometer techniques were used and substrates were added as neutral solutions, to give a final volume of 3 ml per vessel.

Analyses

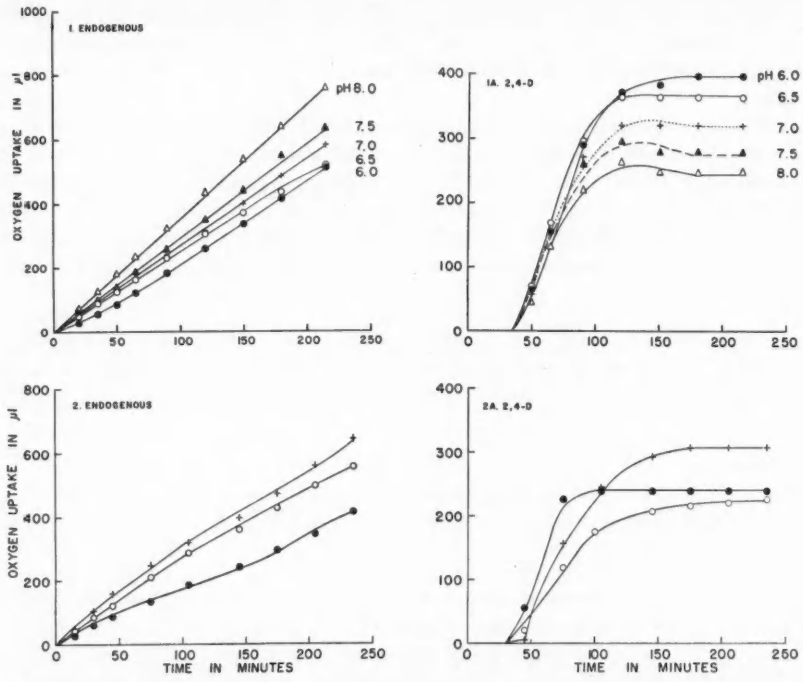
Qualitative analyses were done routinely on metabolized substrates with a Beckman Direct Transmittance Recording Spectrophotometer, Model DK, and quantitative analysis of 2,4-D was done by a chromotropic acid method (12) involving a preliminary ether extraction, after cells were removed by centrifugation.

Results

Oxidation of 2,4-D

The 2,4-D oxidizing system appeared to be quite stable because resting cells were still highly active after storage for 3 weeks at 4° C but neither lyophilized cells nor cell juices were able to oxidize 2,4-D or 2,4-DCP.

Like other investigators in this field, we had somewhat arbitrarily assumed that phosphate buffer, pH 7.0–7.2 and 30° C, would give optimum conditions for the oxidation of the substrates but the validity of these assumptions was not checked until recently. The pH, buffer system, and temperature in the Warburg vessels were varied and the results are shown in Figs. 1, 2, and 3 respectively. Endogenous respiration increased (Fig. 1) whereas respiration due to 2,4-D oxidation decreased (Fig. 1A) with increasing pH values. Of the three buffers tested, barbital proved best for both endogenous (Fig. 2) and substrate (Fig. 2A) respiration but the higher initial rate of oxidation of 2,4-D in the presence of Tris buffer is noteworthy (Fig. 2A). Comparison of the efficacy of the buffers at pH 7.6 instead of pH 7.2 showed similar but less pronounced differences. Endogenous respiration increased linearly with time over the range 25–35° C whereas the 2,4-D oxidizing system showed an optimum temperature of ca. 30° C (Fig. 3).



FIGS. 1 and 1A. Effect of initial pH on endogenous respiration and on oxidation of 2,4-D (2 µmoles). Fig. 1A values corrected for endogenous. Cells in 0.05 *M* KH_2PO_4–Na_2HPO_4 buffer, final concentration.

FIGS. 2 and 2A. Effect of buffer system at initial pH 7.2 on endogenous respiration and on oxidation of 2,4-D (2 µmoles). Fig. 2A values corrected for endogenous. Cells in 0.025 *M* buffer, final concentration.

+—+ barbital; ●—● tris; ○—○ phosphate.

Despite the fact that only one of the original assumptions was substantiated, manometric experiments were not repeated because the results allowed comparison with other work on 2,4-D metabolism.

Although cells were always able to decompose 2,4-D, batches of cells did differ in their respiratory activity (both rate and final amount), in agreement with the findings of Steenson and Walker (19). Two sets of results (one published (6)) which illustrate these differences are given as percentages of oxygen required for theoretically complete oxidation: 2,4-D, 82, 135; MCPA, 67, 100; 2,4,5-T, 33, 44; and 2,4-DCP, 169, 300. Steenson and Walker (19) attributed a similar type of variation to the removal of essential cofactors by washing or to nutritional deficiencies of the growth medium. We have recently found that water washing of the cells can eliminate respiratory activity using 2,4-D after six to seven 50-ml aliquots, which suggests that the amount of washing is critical. It would probably also be useful to study the oxidative properties of cells harvested at various times.

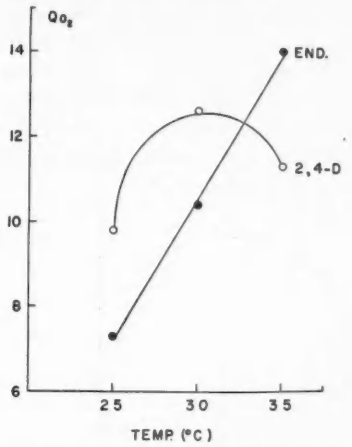


FIG. 3. Effect of temperature on endogenous respiration and on oxidation of 2,4-D (2 μmoles). 2,4-D values corrected for endogenous. Cells, 25 mg dry weight/vessel, in 0.05 *M* phosphate buffer, pH 7.

The percentages of oxidation shown above indicate that some of the compounds or their oxidation products stimulated endogenous respiration, and therefore stoichiometric calculations must be based on the amount of substrate actually decomposed, as determined by analysis. If 2,4-D acts as an uncoupling agent, it would be expected to cause the release of orthophosphate under suitable conditions but we have been unable to find a release of phosphate from cells exposed to the herbicide. It was thought possible that cations from the neutralization of acids might have stimulated metabolism but solutions of Na^+ equimolar to the substrates used herein did not affect the endogenous respiration. However, preliminary results did indicate that the endogenous metabolism could be increased by the classical uncoupling agent 2,4-dinitrophenol at $10^{-3} - 10^{-4}$ *M*.

Maximum uptake of oxygen was observed when 10–15 μmoles of 2,4-D were added to 20 mg (dry weight) of cells, an effect possibly due to enzyme saturation or substrate toxicity. Rogoff and Reid (14) found that 2,4-D was toxic at levels above ca. 3.8 μmoles.

The results given in Fig. 4 suggest that the oxidation of 2,4-D is accomplished by the action of induced enzymes. There appeared to be a fairly brief but clear lag period before 2,4-D was oxidized by non-adapted cells whereas 2,4-D was oxidized immediately by adapted cells. In contrast to 2,4-D, 2,4-DCP was oxidized immediately by both adapted and non-adapted cells. The formation of induced enzymes is further suggested by the fact that 200 p.p.m. NaN_3 inhibited the decomposition of 2,4-D by an otherwise effective soil population but it did not inhibit the decomposition by adapted *Achromobacter* cells (4). This effect of azide might be explained by the findings of

Sussman and Spiegelman (22) which showed that azide inhibited both the appearance and disappearance of induced enzymes probably by reducing competitive interactions among synthetic systems.

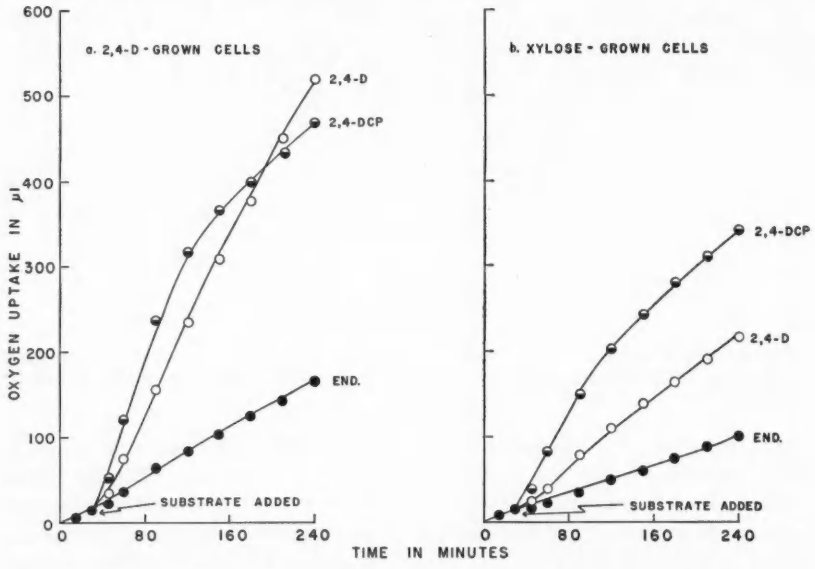


FIG. 4. Oxidation of 2,4-D (5 µmoles) and 2,4-DCP (2 µmoles) by adapted (*a*) and non-adapted (*b*) cells. Cells, 7.6 mg dry weight/vessel, in 0.066 *M* phosphate buffer, pH 7. Non-adapted cells grown in medium 23YM containing 0.1% xylose instead of 2,4-D.

Anaerobic Reduction of Methylene Blue

Conventional Thunberg techniques were used to determine if resting *Achromobacter* cells could dehydrogenate 2,4-D and some of its analogues, and the results are shown in Table I. The rate and degree of reduction substantiate, for the most part, the results given in Figs. 5 and 6 and by Bell (6),

TABLE I

Anaerobic reduction of methylene blue by resting cells of *Achromobacter* sp. grown in medium 23YM

Substrate (0.1 moles/4 ml)	% reduced*	Time (min)
2,4-D	80–90	35
MCPA	60–70	60
2-Cl-PAA	60–70	60
4-Cl-PAA	60–70	60
2,4,5-T	60–70	60
PAA	60–70	180
2,4,6-T	0	180
None	0	180

*Determined visually on duplicate tubes.
Incubation at 28° C in 0.066 *M* phosphate buffer, pH 7.

although 2,4,5-T was oxidized more rapidly than was expected. The failure to dehydrogenate 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) might indicate that its low rate of aerobic oxidation was caused by enzymes other than methylene-blue-reducing dehydrogenases. The rate of dehydrogenation might depend upon the permeability of the cell to the particular substrate or perhaps the substrate or one of its metabolites might stimulate endogenous dehydrogenases.

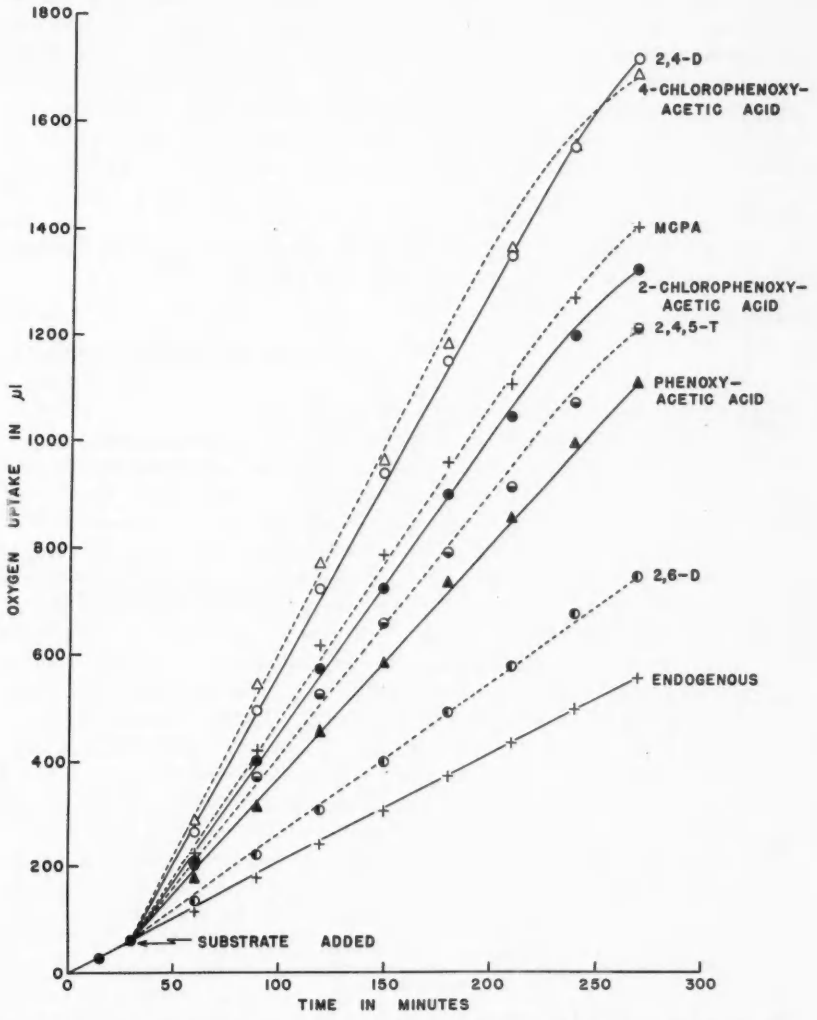


FIG. 5. Oxidation of analogues and derivatives of 2,4-D by *Achromobacter* sp. Substrate 10 µmoles/3 ml 0.066 *M* phosphate buffer, pH 7: cells 20 mg dry weight/vessel: 2,4-D-grown cells.

Substrate Specificity of 2,4-D-adapted Cells

Figures 5 and 6 show the ability of 2,4-D-grown *Achromobacter* cells to oxidize 2,4-D and some of its analogues and derivatives. Comparison of the rate of oxidation of 2,6-D with those of 2,4-D and 6-OH-2,4-D suggests that a readily reactive ortho position is required for the degradation of the aromatic moiety of 2,4-D. The small, net oxygen uptake with 2,6-D might have been due to the oxidation of impurities but more likely to the oxidation of the aliphatic side chain (cf. phenylacetic acid, Fig. 6). The loss of 2,6-D which could not be attributed to adsorption was verified by analysis. In another experiment, cells appeared to oxidize 2,4,6-T slowly but interpretation of the

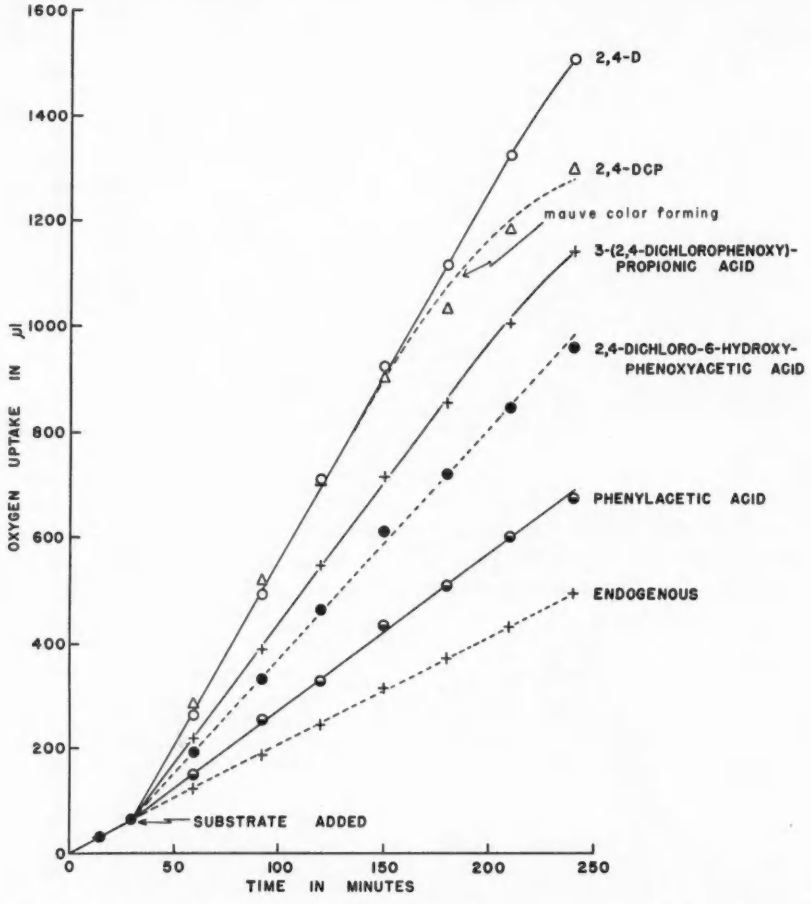


FIG. 6. Oxidation of analogues and derivatives of 2,4-D by *Achromobacter* sp. Substrate 10 µmoles/3 ml 0.066 *M* phosphate buffer, pH 7: cells 20 mg dry weight/vessel: 2,4-D-grown cells.

results is complicated by the fact that increased chlorine substitution inhibits degradation, as occurred with 2,4,5-T (Fig. 5 and references 6, 13, and 24).

The increased respiration of 4-Cl-PAA over that of 2-Cl-PAA and PAA (Fig. 5) might indicate that the para chlorine is a cardinal structural requirement. Previous results (6) showed that PAA inhibited growth in 2,4-D media and it might prove to be an effective antimetabolite of 2,4-D. Rogoff and Reid (14) and Walker and Newman (24) also found that their isolates could slowly oxidize PAA and the former workers found the rate of oxidation almost similar to that of 2-phenoxyethanol. Washed 2,4-D-adapted cells of our *Achromobacter* respired 405 μl of O_2 in 3 hours, but in the presence of 2-(2,4-dichlorophenoxy)ethanol (2,4-DE) (2 μmoles/3 ml) the respiration was reduced to 347 μl of O_2 representing a 14% inhibition of endogenous respiration by the substrate. These results suggest that a carboxyl group is necessary for the oxidation of the aryloxy compounds by this organism. However, it does not appear absolutely essential for utilization that the substrate have a carboxyl group β to the ethereal oxygen because 3-(2,4-D)P was readily oxidized, although more slowly than 2,4-D (Fig. 6). In contrast, Vlitos (23) presented evidence indicating that some soil organisms could convert 2,4-DE to 2,4-D.

Behavior of Possible Intermediates

2,4-Dichlorophenol

Audus (2) suggested that the initial step in the degradation of 2,4-D was the hydrolytic cleavage of the ethereal linkage to form 2,4-DCP and glycolic acid. The formation of 2,4-DCP has been further indicated by the results of Rogoff and Reid (14) and Walker and Newman (24), who found that their 2,4-D-decomposing bacteria could also oxidize 2,4-DCP but they did not repeat the work using cells grown in the absence of 2,4-D. Steenson and Walker (20) showed that young cultures of *Achromobacter* sp. could oxidize 2,4-DCP only if they were grown in the presence of 2,4-D, thus strongly suggesting the intermediary role of 2,4-DCP. Furthermore, Bell (6) demonstrated both the cellular oxidation of 2,4-DCP and its presence in cultures containing 2,4-D and 0.1% (w/v) yeast extract. However, the 2,4-DCP may not have been a "normal" metabolite. Steenson and Walker (21) proposed that 2,4-DCP and 5-chloro-2-cresol were the initial oxidation products of 2,4-D and MCPA respectively. They also found that 2,4-DCP could induce the formation in *Achromobacter* sp. of enzymes oxidizing 2,4-D.

Thus, most evidence indicates that 2,4-DCP is likely to be an intermediate in the bacterial degradation of 2,4-D but application of Stanier's technique of simultaneous adaptation (16) revealed that non-adapted (to 2,4-D) cells of our *Achromobacter* could also vigorously oxidize 2,4-DCP without an appreciable lag period (Fig. 4). The results seem to indicate that the organism has so-called "constitutive" enzymes able to decompose 2,4-DCP. "Boiled" cells did not oxidize 2,4-DCP.

The most favorable concentration for the oxidation of 2,4-DCP was 2–5 μmoles per 3 ml when 20 mg dry weight of cells was used, although there

appeared to be a definite proportion of cells to substrate required for maximum activity. In addition, for unknown reasons, some aliquots of washed cells oxidizing 2,4-DCP would turn mauve-pink coincident with a drop in oxygen consumption (Fig. 6).

2,4-Dichloro-6-hydroxyphenoxyacetic Acid and 3,5-Dichlorocatechol

Evans and Smith (9) suggested that 2,4-D might be decomposed via 6-OH-2,4-D and 3,5-DCC but Steenson and Walker (20) found that their *Achromobacter* sp. could not oxidize either compound. It is apparent from Fig. 6 that our *Achromobacter* can oxidize 6-OH-2,4-D although not so well as 2,4-D. The slower rate of oxidation may have been caused by permeability barriers but even so, no intermediary role can be assigned to this compound until it is found to be oxidized only by 2,4-D-adapted cells or preferably, when it is isolated following the metabolism of 2,4-D.

No oxygen uptake has been demonstrated with 3,5-DCC as substrate but the compound did appear to be metabolized by washed cells. In one respect the organism acted similarly on 3,5-DCC and 2,4-DCP in that some batches of cells turned the 3,5-DCC mauve-pink, coincident with an inhibition of endogenous respiration. "Boiled" cells (6) from these batches did not do this. It is tempting to suggest that some cells produced a toxic substance, possibly a quinone, similar to that which was produced from 2,4-DCP.

2,4-Dichloroanisole

No one seems to have considered 2,4-dichloroanisole (2,4-DA) as a possible intermediate in the bacterial dissimilation of 2,4-D but it is a likely possibility (Fig. 7) considering the ubiquity of decarboxylases. Unfortunately this compound is difficult to use because of its insolubility in water and its volatility, but exposure of 2,4-D-grown cells to 2,4-DA vapors in Warburg vessels resulted in a net uptake of 637 μl O_2 in 3 hours, indicating that it might be an intermediate in 2,4-D metabolism. Non-adapted cells were not tested. Attempts to demonstrate the anaerobic decarboxylation of 2,4-D to 2,4-DA failed.

Oxidation of Carboxylic Acids and Pentoses

It has been shown (6) that the dicarboxylic acids of the tricarboxylic acid cycle are amongst the best growth stimulants and it was therefore of interest to examine the capacity of the *Achromobacter* sp. to oxidize several members

TABLE II

Oxidation of carboxylic acids by resting cells of *Achromobacter* sp. grown in medium 23YM

Substrate (10 μmoles)	Q_{O_2}*	% oxidized†
Succinate	5.2	40
Acetate	2.9	34
Fumarate	3.4	26
Pyruvate	2.5	24
Malate	2.9	22
α-Ketoglutarate	2.9	17

*Endogenous subtracted. Cells 14 mg dry wt./vessel, 0.066 *M* phosphate buffer, pH 7.
†Based on amount of O_2 required for complete oxidation. At the end of 220 min, pyruvate oxidation only had returned to endogenous level.

of the cycle. Apparently the oxidative utilization of the substrates shown in Table II does not bear any direct relationship to their effectiveness as growth stimulants but it may be that their efficacy lies in their ability to "spark" metabolism or to supply necessary intermediates. It is also possible that the method of utilization of the substrates by resting and growing cells may differ. The cells utilized 2,4-D much more rapidly than any of the carboxylic acids tested.

It has been shown also (6) that pentoses were utilized by the *Achromobacter* sp., so they too were examined manometrically with the following results; arabinose Q_{O_2} 1.3, ribose Q_{O_2} 1.4, lyxose Q_{O_2} 0.7, and xylose Q_{O_2} 1.0. Apparently the pentoses were oxidized to a slight extent (net) and as with the acids the rates of oxidation had not approached that of the endogenous at the end of 220 minutes. Both adapted and non-adapted (to xylose) cells oxidized xylose at the same slow rate.

Discussion

It has been established that the *Achromobacter* sp. studied here oxidizes 2,4-D, probably by forming induced enzymes, and there is little doubt that it also oxidizes a variety of other chlorinated, aromatic compounds. These seemingly catholic tastes have made it difficult to determine the mechanism of degradation of 2,4-D but suggestive results have accrued.

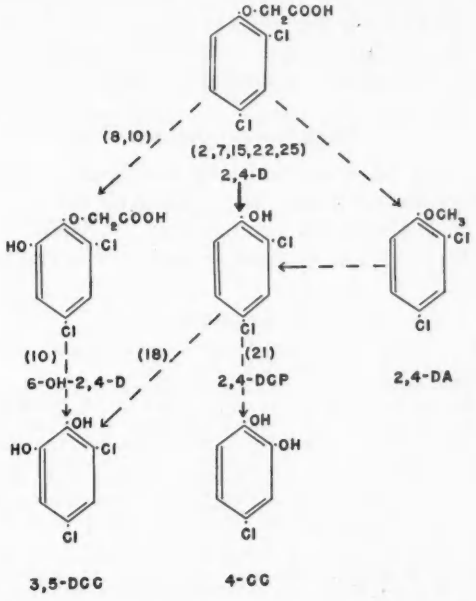


FIG. 7. Proposed and probable pathways in the bacterial degradation of 2,4-D. Broken arrows indicate proposed routes; solid arrow probable route. Numbers in parenthesis indicate pertinent references. Abbreviation 4-CC, 4-chlorocatechol.

The ability of cells to dehydrogenate 2,4-D as shown by the reduction of methylene blue might indicate that pyridine nucleotide coenzymes rather than oxidases or peroxidases are operative, at least in the initial stages of degradation.

It is possible that 6-OH-2,4-D might be an intermediate in the decomposition of 2,4-D but 3,5-DCC does not appear to be, although at times the metabolites of 3,5-DCC and 2,4-DCP reacted similarly, suggesting that 2,4-DCP might be decomposed via 3,5-DCC. The intermediary role of a catechol is quite probable because ring fission is often preceded by oxidation at adjacent positions on the ring (17). Further work must be done to account for the variation in behavior between batches of cells, bearing in mind the toxicity and instability of mono- and poly-hydroxy phenols and their probable low physiological concentration.

It is still uncertain whether or not 2,4-DCP is an intermediate in the dissimilation of 2,4-D by our *Achromobacter* because the phenol is oxidized by both non-adapted and adapted cells. Therefore, techniques other than that of simultaneous adaptation (sequential induction) must be employed to solve this problem, although this technique may aid in determining the role of other suspected intermediates. The use of compounds which can block 2,4-D degradation at various steps or trap the products, or of partially incapacitated cells (e.g. acetone-treated) and cell juices might prove fruitful.

In resumé, the proposed and probable pathways of 2,4-D metabolism by various bacteria are given in Fig. 7. It is quite possible that each organism which can degrade phenoxyacetates does so via unique pathways but the basic mechanisms involved may be much the same.

Acknowledgments

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THE EFFECT OF PARTIAL STERILIZATION OF FIELD SOILS BY CHEMICALS UPON DIFFERENTIAL MICROBIAL COUNTS, CO_2 ACTIVITY, AND RATES OF ORGANIC MATTER DECOMPOSITION[1]

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Abstract

Varying dosage rates of calcium hypochlorite, mercuric chloride, and sodium propionate were used as partial sterilization agents in field and greenhouse trials to determine the effect on soil biological activity, and thereby, organic matter decomposition rates. Application of the chemicals often resulted in the initial stimulation of respiration rates. In greenhouse and field trials this stimulation was most commonly associated with the application of sodium propionate. Fungal counts decreased and bacterial counts increased when this chemical was used. Within 4 weeks after treatment, all rates of application of the various chemicals depressed soil CO_2 evolution. Thirteen months after treatment, plots treated with 100 lb of mercuric chloride per acre had one quarter as many actinomycetes and total microbes, and one third as many fungi, as did the control plots. Respiration was also considerably lower. Calcium hypochlorite treated plots retained reduced total counts and sodium propionate reduced fungal counts. Respiration in these last plots was not depressed.

The changes in organic matter decomposition due to chemical treatments were not statistically significant in the greenhouse or field studies.

General lack of correlation between the three measures of biological activity used in this study indicate the necessity for the further development of methods for measuring the population of microorganisms in soil.

Introduction

Soil organic matter is rapidly decomposed by microbial activity in areas having warm, humid climates. Theoretically, if the microbial population, or the activity of that population, were to be reduced, then the rate of organic matter decomposition should also be reduced. The effects of partial sterilization of soil upon crop growth, microorganisms, and the physical and chemical constituents of soil have been studied for more than 65 years. A detailed review of investigations through 1917 has been written by Kopeloff and Coleman (9). Further reviews were written by Waksman and Starkey in 1923 (15, 16, 17) A short anonymous review appeared in 1948 (2). Extensive reviews by Hall and Clegg in 1949 (6), and Warcup in 1957 (18), discuss the results of investigations concerned with partial sterilization of soil. Several workers have reported that partial sterilization by steam resulted in higher levels of soluble organic material in soil (1, 8, 11, 12, 14). Waksman and Starkey (17) found that partial sterilization by both steam and chemicals made soil organic matter more available to decomposition by microorganisms.

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In spite of the widespread interest in partial sterilization of soil, little further work has been reported concerning the effect of partial sterilization upon the soil organic matter.

The purpose of this paper is to present data showing the influence of partial sterilization upon the rate of organic matter decomposition in soil, and the effects of such treatment upon the numbers and activity of the soil microorganisms. Data are presented to correlate the three measures of soil activity: microbial numbers, CO_2 production, and organic matter decomposition.

Materials and Methods

Three chemicals with widely divergent antimicrobial activity were chosen as sterilization agents. Mercuric chloride was selected as a heavy metal poison with long-term residual activity. Conversely, calcium hypochlorite was used because of its non-persistence. Since fungi are believed to be important agents in the decomposition of organic matter, sodium propionate was selected as a fungicide. All three chemicals were applied in powder form to experimental plots. Supplemental irrigation was then used to bring the compounds into solution and distribute them in the upper soil profile.

Differential plate counts were determined using Bhat's medium (3) for total microbial numbers, Jensen's (7) for the actinomycetes, and Martin's (10) rose bengal agar for soil fungi. Martin's medium was modified by the deletion of streptomycin since the absence of this component did not appear to affect the selectivity of the medium.

Since numbers of microorganisms in soil may not always give a direct measure of the activity of that population, a rapid, reproducible method was developed for measuring biological activity as CO_2 evolution. Ten grams of sieved, fresh soil were added to 125-ml Erlenmeyer flasks containing center wells. Each flask was closed with a serum stopper and incubated at 30° C for a period of from 2 to 15 hours, depending upon the activity of the sample. After a suitable incubation time, 1 ml of approximately .04 *N* $Ba(OH)_2$ was added to the center well through the stopper by means of a syringe. The flasks were then shaken for 45 minutes to aid absorption of the CO_2 by the alkali. Respiration was measured quantitatively by titrating the excess alkali directly in the center well with .02 *N* HCl, using a microburette. Respiration was recorded in terms of milliequivalents of CO_2 evolved per hour and calculated on the basis of 1 g of oven-dried soil. A blank was used to correct for the atmospheric CO_2 present in the flasks. As many as 36 samples were conveniently run simultaneously with exceptionally reproducible results. The technique has several obvious advantages. Fresh soil samples at the field moisture levels are utilized. The usually short incubation time minimizes changes in the microbial activity which arise due to the changing environmental conditions resulting from soil sampling. Further, the atmosphere within the flask is nearly that of normal air, so that enough CO_2 is initially present to supply the level required by many microorganisms.

For organic matter determinations, the potassium dichromate, oxidation–reduction, titration method of Peech *et al.* (13) was used.

Field plots 2 ft by 3 ft in size were established on an old permanent pasture of Groseclose silt loam (field 1). All treatments were replicated three times in a random block design. Alternate plots were treated so that a buffer strip 2 ft by 3 ft remained between each treated plot. Each block was separated from the next treated block by a 3-ft buffer zone which was established for the purpose of preventing mixing and consequent interactions between the various chemicals. Calcium hypochlorite, mercuric chloride, and sodium propionate were initially applied at rates of 5 and 10 lb per acre during August, 1956. Each plot was re-treated 21 days later with the same chemical but with 50 or 100 lb per acre.

Another set of plots was established on a newly planted sod which had been seeded on Groseclose silt loam soil in the spring of 1957 (field 2). The previous cover had consisted of a dense growth of weeds and mixed grasses. A random block design consisting of four replicates of plots 3 ft by 5 ft in size was utilized. Again, alternate plots and blocks were treated. Each of the three chemicals was applied on separate plots at rates of 5, 25, 50, 100, and 200 lb per acre in July, 1957.

A series of greenhouse plots was established on a bluegrass – white clover sod in a greenhouse bench containing soil to a depth of 12 in. Unsterilized soil from the vicinity of field 2 was used. Three replications of plots, 1 ft by 2 ft in size, were arranged in random block design. Alternate plots and blocks were treated with 50 and 100 lb of each chemical per acre. Two sets of blocks were established: The first set was treated with chemicals in December, 1956; the second set was treated in February, 1957, and re-treated in April, 1957.

The general procedure for sampling field soil was as follows: Three $\frac{3}{4}$-in. diameter plugs of soil were taken at random from each plot to a depth of 4 in. In the greenhouse, however, an 8-mm diameter cork borer was used to sample to a depth of 4 in. The plugs in each case were combined as one plot sample.

When CO_2 evolution was used to measure the activity of the microbial population in response to chemical treatment, duplicate flasks were run for each plot sample. In later studies, the samples from all replicates of the same treatment were pooled.

Differential plate counts of treated soils were obtained periodically. Samples for plate counts were deflocculated with NaCl as described by Wilson (19). The technique was that of Gamble (5). Duplicates of each sample were diluted to the appropriate count range, and plated in triplicate on each of the differential media. Organisms were counted after incubation at 30° C for 7 days.

Results

It was frequently observed that treatment of soil with various levels of sodium propionate initially resulted in increased CO_2 evolution. Tumbler cultures indicated that, together with increased CO_2 production, a marked shift in the microbial ecology was attributable to sodium propionate. Plate

counts of samples 7 days after treatment, equivalent to 100 lb of sodium propionate per acre, showed a 25% increase in total counts when compared with untreated control tumblers. Fungal counts were 75% lower in the treated tumblers, and actinomycete counts were 8% lower. The results of greenhouse and field trials, as shown in Table I, demonstrate that 3 days after treatment, 50 and 100 lb of sodium propionate per acre again resulted in higher total counts while the fungi numbers were reduced.

TABLE I

Response of differential microbial counts to applications of 50 and 100 lb of calcium hypochlorite, mercuric chloride, and sodium propionate per acre at various intervals after treatment and in various locations (counts reported as percentage of control value)

Chemical treatment, lb/acre		Sampling interval after treatment			
		Greenhouse		Field No. 2, 90 days	Field No. 1, 13 months
		3 days	15 days		
		Total microbes			
Control		76.0×10^5	93.0×10^5	13.7×10^6	28.4×10^5
$Ca(OCl)_2$	50	377.6%	83.9%	57.7%	
	100	113.2%	100.0%	83.9%	62.0%
$HgCl_2$	50	69.7%	112.9%	44.5%	
	100	76.3%	64.5%	39.4%	26.1%
$NaC_3H_5O_2$	50	178.9%	133.3%	88.3%	
	100	226.3%	143.0%	56.9%	97.9%
		Fungi			
Control		8.1×10^5	15.0×10^5	4.9×10^5	14.2×10^4
$Ca(OCl)_2$	50	140.1%	60.0%	26.5%	
	100	76.5%	53.3%	42.9%	88.7%
$HgCl_2$	50	35.8%	93.3%	69.4%	
	100	34.6%	40.0%	67.3%	40.1%
$NaC_3H_5O_2$	50	25.9%	126.7%	34.7%	
	100	16.0%	93.3%	44.9%	66.9%
		Actinomycetes			
Control		26.0×10^6	59.0×10^6	14.7×10^5	23.9×10^5
$Ca(OCl)_2$	50	157.7%	149.2%	58.5%	
	100	111.5%	125.4%	101.4%	101.7%
$HgCl_2$	50	23.1%	127.1%	84.4%	
	100	30.8%	100.0%	55.1%	27.2%
$NaC_3H_5O_2$	50	107.7%	144.1%	76.9%	
	100	142.3%	140.7%	79.6%	132.6%

Similar levels of mercuric chloride generally decreased all differential counts. This effect persisted throughout the experiment. Calcium hypochlorite initially stimulated all three groups of microorganisms. This stimulation was reversed in later observations. Carbon dioxide activity, as illustrated in Table II, was apparently well correlated with total count data for these plots. CO_2 activity as reported in the table was determined from samples obtained simultaneously from the same plots as those reported in Table I.

Usually within 30 days after treatment, plots treated with each of the chemicals exhibited lower microbial activity as measured by differential counts and CO_2 production than did the control plots. This lowered activity

was found to be persistent. Thirteen months after treatment in field No. 1, statistically significant effects due to treatment were measured for total and fungal counts as well as CO_2 evolution. These data are summarized in Tables I, II, and III.

TABLE II

CO_2 activity of soil samples collected at various times after treatment (reported in meq of CO_2/hour per gram of dry soil)

Chemical treatment, lb/acre		Sampling interval after treatment			
		Greenhouse		Field No. 2, 90 days	Field No. 1, 13 months
		3 days	15 days		
Control		12.5×10^{-5}	17.9×10^{-5}	17.9×10^{-5}	15.5×10^{-5}
$Ca(OCl)_2$	50	14.2 "	20.8 "	15.0 "	13.1 "
	100	14.2 "	19.4 "	17.9 "	16.0 "
$HgCl_2$	50	10.6 "	27.8 "	14.8 "	13.8 "
	100	12.0 "	19.4 "	20.3 "	11.6 "
$NaC_3H_5O_2$	50	13.9 "	17.9 "	15.7 "	15.5 "
	100	16.7 "	22.0 "	16.9 "	15.5 "

TABLE III

Statistical summary evaluating the effects of chemical treatment upon total, fungal, and actinomycete counts; organic matter level; and CO_2 activity

Source of variation	Mean squares				
	Total	Fungi	Actino-mycetes	% organic matter	CO_2 activity
	Greenhouse plots 3 days after treatment				
Between treatments	50.52**	20.32**	342.71**		
Error	9.01	3.13	12.33		
	Field plots (field No. 2) 90 days after treatment				
Between treatments	2,620.78*	413.93	3,139.70**	.436	.327**
Error	511.20	134.50	120.05	.289	.045
	Field plots (field No. 1) 13 months after treatment				
Between treatments	419.34*	84.38**	585.05	.162	7.57**
Error	17.94	3.12	91.50	.097	.378

*Significant at the 5% level of probability.
**Significant at the 1% level of probability.

Plots in field No. 2, treated with 100 lb of the three chemicals per acre, were sampled at frequent intervals for 90 days for the purpose of determining CO_2 activity in these treated soils. As shown in Fig. 1, the CO_2 activity following treatment in this field did not follow the pattern found in either the greenhouse or the permanent pasture plots (field No. 1). As might be expected, the moisture content of the soil appeared to greatly influence the CO_2 activity of the control plots. A similar comparison could be made between moisture content and the calcium hypochlorite and sodium propionate curves. There appeared to be a strong interaction between soil moisture and the intensity of chemical treatment effects upon CO_2 activity in the soil.

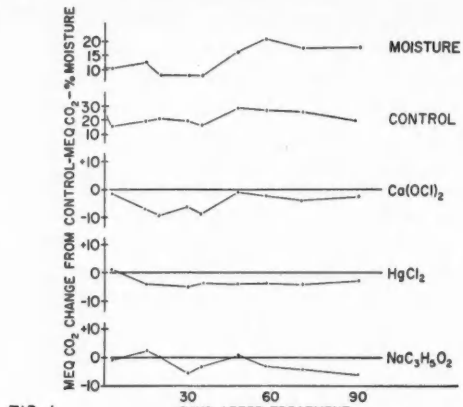

FIG. 1

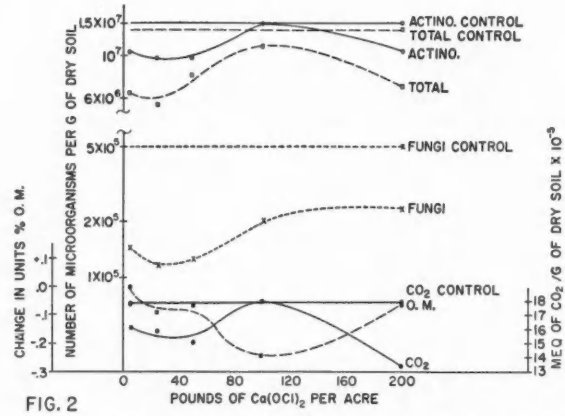

FIG. 2

FIG. 1. Effect of 100 lb calcium hypochlorite, mercuric chloride, and sodium propionate per acre, on field soil CO_2 evolution with respect to time after treatment (field No. 2).

FIG. 2. Effect of applications of calcium hypochlorite upon microbial counts, CO_2 evolution, and organic matter decomposition (field No. 2) (sampled 29 Oct. 1957, 90 days after treatment).

Plots in field No. 2, treated with 5, 25, 50, 100, and 200 lb of calcium hypochlorite per acre, were sampled after 90 days for differential microbial counts, CO_2 evolution, and organic matter decomposition. Figure 2 summarizes these results. Similar data for mercuric chloride and sodium propionate treated plots are presented on Figs. 3 and 4, respectively. Statistically significant effects upon total and actinomycete counts, as well as upon CO_2 activity, were found to have resulted from treatments with the chemicals. A highly significant positive correlation was measured between total counts and organic matter levels on the mercuric chloride treated plots, while a significant negative correlation was measured between these same factors when the plots were treated with sodium propionate. A highly significant positive correlation was

measured between CO_2 activity and organic matter levels when the soil was treated with calcium hypochlorite. The only significant correlation between CO_2 evolution and total plate counts was observed on mercuric chloride treated soils. There appeared to be no consistent statistical correlation between count data, CO_2 evolution, and organic matter level at increasing rates of chemical application after this 90-day period.

Residual organic matter was determined at various times after treatment in both fields and in the greenhouse plots. No statistically significant change in percentage organic matter decomposition could be attributed to chemical treatment in any of the plots.

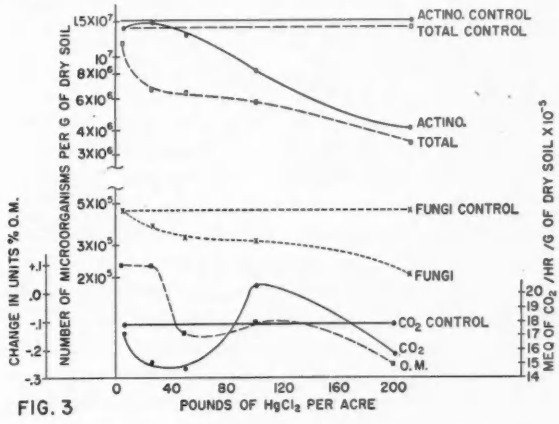

FIG. 3

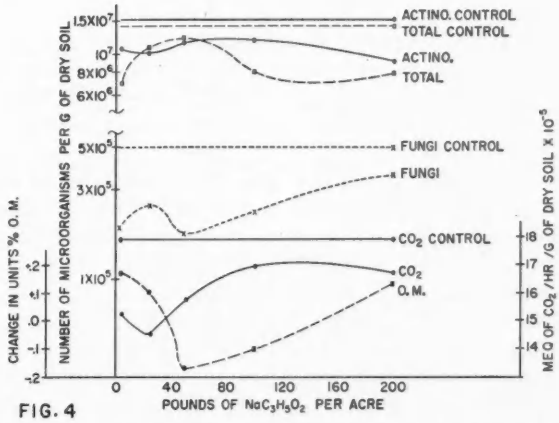

FIG. 4

FIG. 3. Effect of applications of mercuric chloride upon microbial counts, CO_2 evolution, and organic matter decomposition (field No. 2) (sampled 29 Oct. 1957, 90 days after treatment).

FIG. 4. Effect of applications of sodium propionate upon microbial counts, CO_2 evolution, and organic matter decomposition (field No. 2) (sampled 29 Oct. 1957, 90 days after treatment).

No attempt was made to evaluate the effect of partial sterilization upon plant growth, but qualitative observations indicated considerable chlorosis and growth inhibition resulting from the application of 200 lb of mercuric chloride per acre. A less pronounced chlorotic condition, and perhaps some growth inhibition, was noted in plots treated with 100 lb of mercuric chloride or 200 lb of calcium hypochlorite per acre.

Because of the large numbers of determinations involved in plating for microbial counts, replicate plot samples were pooled in a composite sample. This eliminated a measure of the block effect and sacrificed statistical precision, since this pooling assumed that there was no block × time interaction. In CO_2 activity measurements, however, such an interaction was tested for and did not prove to be statistically significant.

Discussion

It has been previously reported that partial sterilization of soil by either steam or chemical means often results in a temporary decrease in microbial numbers and activity which is followed by a variable period of considerably increased numbers of microbes. The results of this preliminary study, both in greenhouse and field, are not in agreement with previous observations. In all cases there resulted a long period of lowered CO_2 activity which often followed a short temporary increase in both number and activity, notably when sodium propionate was used. This lengthy duration of inhibitory effects indicates that such chemical treatment under some field conditions may be a promising means for controlling microbial ecology and total numbers in the soil. Further work is needed to establish the types of chemicals as well as application rates needed for the desired control.

The changes in organic matter levels due to treatment are not statistically significant. Since microorganisms are responsible for decomposition of organic residues, and since such chemical agents as mercuric chloride proved effective in reducing fungal, actinomycete, and total microbial counts, as well as respiration rates for a considerable length of time, it would appear logical that such an agent should also change the rates of organic matter decomposition. No consistent significant correlation, however, was found among the microbial count data, CO_2 evolution, and organic matter decomposition.

It is possible that some agent active in the decomposition of organic matter may not have been detected with the techniques used in this study. Present plating techniques used for estimating the microbial population of soil assume that a small but constant fraction of the total numbers is counted. It is possible that available media do not support certain groups of bacteria, terrestial fungi, and actinomycetes which are important in the decomposition of organic matter. No satisfactory methods exist for estimating the numbers of algae, protozoa, and aquatic phycomycetes present in the soil, which undoubtedly also contribute to the total CO_2 activity. These and other such groups probably play important roles in soil processes and so should be considered in estimates of microbial counts.

The methods for measuring respiration are well refined. Respiration methods which rely upon intact soil samples, such as the method used in this study, may contain inherent errors, since not only the respiration of the microflora is measured but also the often considerable contribution of the micro- and smaller macro-fauna of the soil is included in the estimate of respiration.

The apparent lack of consistent correlation between plate count data and CO_2 activity in soil presents an important barrier to progress in some basic areas of soil microbiology. Improved methods for the estimation of microbial activity in soils appear essential for the determination of the functions, requirements, and interrelationships of the major soil populations.

Acknowledgments

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ÉTUDE D'UNE POPULATION DE BACTÉRIES RÉDUCTRICES DU SOUFRE[1]

R. DESROCHERS ET V. FREDETTE

Abstract

A study has been made of the populations of sulphur-reducing bacteria in a certain section of the Ottawa river.

Not only have the numbers of these populations been determined over a 10-mile section, but also several factors such as dissolved oxygen and biochemical oxygen demand have been examined for their relation with the variations in number of these same populations.

The yield of sulphate-waste liquors in numbers of population supported as well as the amount of hydrogen sulphide produced under natural conditions have also been studied.

Introduction

Les bactéries anaérobies réductrices du soufre, genre Desulfovibrio ou Sporovibrio, sont étudiées depuis la fin du siècle dernier.

L'importance de leur étude réside dans le fait qu'on leur attribue un rôle prépondérant dans la production de l'hydrogène sulfuré à partir des composés inorganiques du soufre: sulfates, sulfites, thiosulfates, etc. En effet, ces vibrions anaérobies ont été constamment retrouvés là où il y avait de l'hydrogène sulfuré produit spontanément: sédiments marins et lacustres, pétroles, égouts, etc.

Leur existence un peu partout dans la nature semble donc confirmée. Quant à leur rôle, on a cherché à s'en faire une idée en dénombrant les populations trouvées dans ces divers habitats; la plupart des travaux effectués dans ce but l'ont été à l'aide de la méthode des dilutions sériées ensemencées en milieu liquide. Ainsi, en 1924, dans une étude concernant une usine d'épuration, Hotchkiss (4), à l'aide d'un milieu minéral, trouva 1000 Desulfovibrio par centimètre cube dans l'affluent, alors qu'il y en avait 100,000 par centimètre cube dans la chambre d'Imhoff, et 10,000 dans l'affluent.

Heukelekian (3) montra qu'il existe une corrélation entre le nombre des bactéries sulfatoréductrices et le taux de production d'hydrogène sulfuré au sein d'égouts emmagasinés ou au repos ("stored sewage").

En 1953, Grossman et Postgate (2) montrèrent que l'addition au milieu de culture d'un agent réducteur, comme la cystéine, permettait de compter à 60% près le nombre d'unités dans une population de bactéries réductrices du soufre.

Drummond et Postgate (1) trouvèrent 7×10^7 Desulfovibrio par 100 cc dans l'eau d'un étang artificiel contaminé. Ces auteurs constatèrent de plus que pour déceler la présence de Desulfovibrio à l'aide d'un milieu sans cystéine il faut une population d'au moins 10^5 germes par 100 cc. Ceci explique les faibles nombres rapportés antérieurement.

[1]Manuscrit reçu le 25 septembre 1959.
Contribution de l'Office de Biologie, Gouvernement de la Province de Québec, et Département de Bactériologie, Faculté de Médecine de l'Université de Montréal, Montréal, Qué. Ce travail a été subventionné partiellement par Canadian International Paper Company.

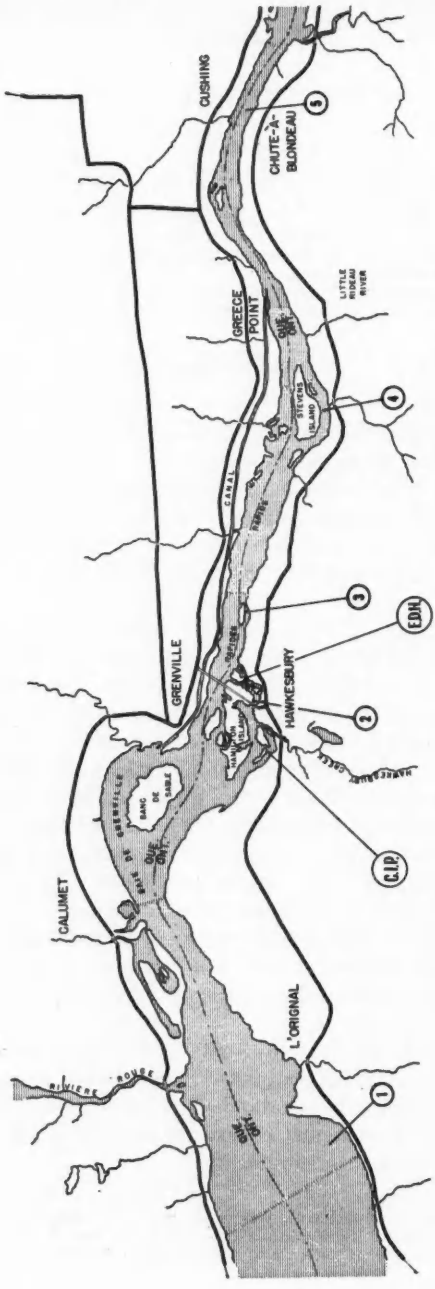

FIG. 1. Section de la rivière Ottawa entre Orignal et Chute-à-Blondeau. Les endroits de prélèvements sont numérotés de un à cinq. C.I.P., usine de la Canadian International Paper. E.D.H., égouts domestiques de la ville de Hawkesbury.

Dans le présent travail, nous avons fait la recherche et le dénombrement de Desulfovibrio dans une section de la rivière Ottawa comprise entre Orignal et Chute-à-Blondeau.

Cette section semblait favorable au développement de ces microbes parce qu'elle reçoit les eaux usées d'une usine de préparation de cellulose (C.I.P.) ainsi que les égouts domestiques de la ville de Hawkesbury (E.D.H.).

Expérimentation

La recherche des Desulfovibrio fut faite en premier lieu à l'aide du milieu Starkey dont la formule est celle proposée dans "Standard Methods for the Examination of Water, Sewage and Industrial Wastes" de l'American Public Health Association. Après 3 semaines d'incubation à la température du laboratoire, ces bactéries furent effectivement trouvées dans les eaux de la rivière Ottawa.

Pour dénombrer ces bactéries, des échantillonnages furent pratiqués à diverses époques de l'année en cinq endroits différents de la rivière Ottawa, échelonnés sur une distance de 10 milles depuis la baie de l'Orignal jusqu'au village de Chute-à-Blondeau (Fig. 1). Après dilutions décimales de 10^{-1} à 10^{-7}, ces échantillons étaient ensemencés le jour même dans le milieu à la cystéine. Voici la composition de ce milieu:

K_2HPO_4	0.5 g	Lactate de soude (solution à 60%)	3.5 g
NH_4Cl	1.0 g	Extrait de levure (Difco)	1.0 g
Na_2SO_4	1.0 g	Cystéine HCl	1.0 g
$CaCl_2.6H_2O$	0.1 g	Sel de Mohr	0.5 g
$MgSO_4.7H_2O$	2.0 g	Eau distillée	1000 cm^3

Ajuster à pH 7.0–7.6

La cystéine et le sel de Mohr sont stérilisés par filtration puis incorporés au milieu.

Après 3 jours d'incubation à 30° C dans des jarres Brewer en atmosphère d'hydrogène, les tubes présentant un précipité noir de sulfure de fer étaient repiqués en milieu sans cystéine afin d'éliminer les bactéries qui n'attaquent que le soufre organique (Drummond et Postgate). Après 3 autres jours dans ce nouveau milieu, les dilutions ayant de nouveau fourni un précipité noir sont alors reconnues comme renfermant des Desulfovibrio et servent par conséquent au dénombrement des populations.

Résultats

Les résultats obtenus au cours de l'année 1957 sont contenus dans la figure ci-jointe (Fig. 2).

On note:

1. Une augmentation considérable du nombre des Desulfovibrio après le déversement des eaux usées de l'usine de la Canadian International Paper au pont interprovincial de Hawkesbury. En effet, les nombres passent de moins de 100 par centimètre cube à plus de 1,000,000 par centimètre cube. Quoique

moins élevée, cette population est encore de l'ordre de 100,000 par centimètre cube après le déversement des égouts domestiques de la ville de Hawkesbury, i.e. aux environs de l'Île Laurin (no 3 sur la carte).

2. Des variations saisonnières dans le nombre des Desulfovibrio à ces divers endroits. Ainsi, par exemple, la plus grande activité est notée en juin, août et septembre; par contre, en mai et novembre, les populations se maintiennent en bas de 1000 par centimètre cube.

3. Augmentation parallèle des nombres de Desulfovibrio et du B.O.D.

En rapport avec cette dernière constatation, l'expérience suivante nous a permis de déterminer si l'apport de substances nutritives, i.e. matières organiques et soufre, constituait le facteur principal de l'augmentation des populations de Desulfovibrio. Des échantillons d'eau en provenance de l'Orignal, de Hawkesbury et de l'Île Stevens furent stérilisés à l'autoclave puis ensemencés

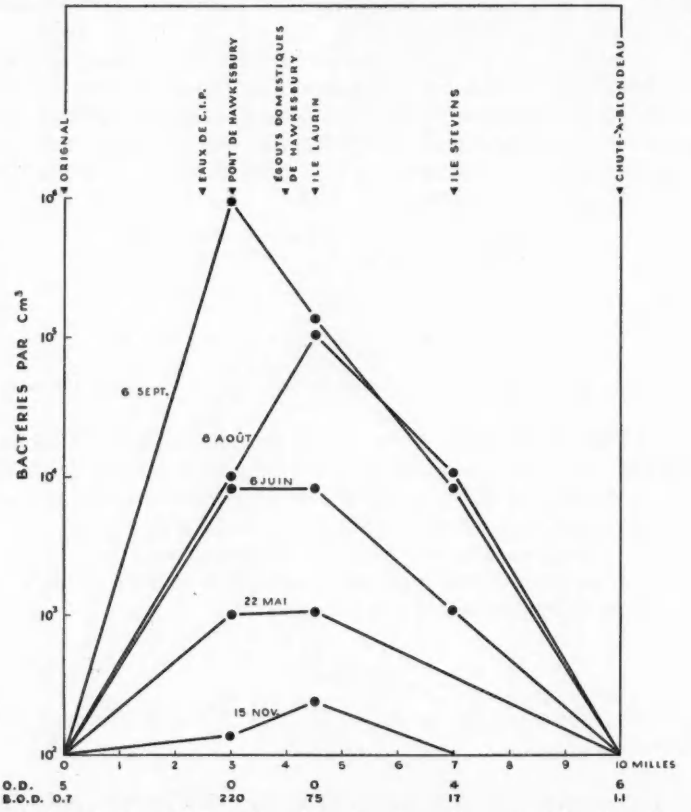

FIG. 2. Distribution des bactéries réductrices du soufre entre Orignal et Chute-à-Blondeau. O.D., oxygène dissout en mg/l. B.O.D., "biochemical oxygen demand". Les mesures de O.D. et de B.O.D. furent effectuées au cours de l'été 1955 (Piché (6)). Les conditions étant identiques en 1955 et 1957, ces mesures varient peu d'une année à l'autre. Des mesures effectuées au cours de l'été 1959 confirment les chiffres trouvés en 1955.

avec une culture de Desulfovibrio. Après 3 jours à 30° C des numérations fournirent les chiffres suivants:

Orignal: 1.1×10^9 (no 1 sur la carte),
Pont de Hawkesbury: 2.7×10^7 (no 2 sur la carte),
Île Stevens: 1.1×10^9 (no 4 sur la carte).

Il s'ensuit que les eaux en question sont susceptibles d'alimenter des populations encore plus grandes que celles que nous avons pu dénombrer dans la nature.

Devant ces faits, il faut penser que les substances nutritives apportées tant par les eaux résiduelles de l'usine que par les égouts domestiques de la ville agissent non pas directement mais surtout comme agents réducteurs capables de conditionner le milieu pour l'établissement d'une certaine population anaérobie de Desulfovibrio. Cette action réductrice peut être à la fois chimique et biologique: les eaux de l'usine sont chimiquement réductrices et, partant, favorables au Desulfovibrio, ainsi que nous l'avons constaté; par contre, les eaux d'égouts domestiques favorisent l'éclosion des bactéries aérobies et facultatives qui contribuent également à la réduction du milieu. Or, ces dernières étant plus actives en été, on comprend que le milieu puisse être réduit à cette époque, d'autant plus que l'oxygène dissous est moins élevé en été qu'en hiver. Ajoutons à cela que les Desulfovibrio ont un optimum thermique supérieur à 20° C, et nous comprendrons facilement les variations saisonnières constatées dans le nombre des Desulfovibrio.

Quel est maintenant le rendement en hydrogène sulfuré de ces populations? Au cours d'une quinzaine de déterminations, nous avons pu déceler H_2S qu'une seule fois, soit le 6 septembre alors que la population atteignait le chiffre-record de 1.1×10^6 Desulfovibrio par centimètre cube. Il faut remarquer toutefois qu'au moment de ce prélèvement l'eau était basse et formait en cet endroit un étang d'où l'eau ne s'écoulait que très lentement. Ce ralentissement du débit de l'eau semble avoir favorisé le développement d'une forte population de Desulfovibrio avec le résultat que l'H_2S devenait alors dosable. Cette hypothèse se trouve appuyée par les travaux de Kimata et ses collaborateurs (5) qui, au Japon, ont noté une relation étroite entre le nombre des bactéries sulfatoréductrices et la teneur des boues marines en H_2S. De même, Pomeroy et Bowlus (7) aux États-Unis rapportent que les égouts qui s'écoulent librement ne donnent pas lieu à un dégagement d'H_2S.

Conclusion

À la lumière des constatations que nous avons pu faire au cours de cette étude préliminaire, il semble que les Desulfovibrio, bactéries anaérobies réductrices des sulfates, sont capables de se développer dans un milieu relativement pauvre (rivière Ottawa en amont de Hawkesbury), où le facteur limitant serait le potentiel d'oxydo-réduction et non pas les substances nutritives sous forme de substances organiques et minérales. D'où il découle que leur intervention directe dans la pollution de l'air et des eaux peut être contrôlée par la modification des facteurs susceptibles d'abaisser le potentiel d'oxydo-réduction.

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A COMPARISON OF ISOLATES OF CLAVICEPS SPP. FOR THE ABILITY TO GROW AND TO PRODUCE ERGOT ALKALOIDS ON CERTAIN NUTRIENTS[1]

W. A. TABER AND L. C. VINING

Abstract

Isolates of *Claviceps purpurea* and *Claviceps* spp. obtained from various geographical areas were compared for their ability to grow and to produce ergot alkaloids in vitro on various carbon and nitrogen sources. While some differences in utilization of carbon sources for growth were found, there was no observed correlation between utilization of carbohydrates and the capacity to produce ergot alkaloids. The amount of alkaloid produced by different strains depended upon both the carbon and nitrogen sources. In general, those cultures capable of alkaloid production were able to do so on more than one carbon source, but the carbon source allowing greatest production differed from one strain to another. Both producing and non-producing strains could utilize succinic acid as a carbon source for growth, but neither could utilize L-tryptophane as a carbon or nitrogen source for growth.

Introduction

A previous survey (11) of 41 isolates of *Claviceps purpurea* (Fr.) Tul. demonstrated that strain differences existed with respect to ability to produce ergot alkaloids on a medium containing mannitol as a carbon source. A more detailed survey has now been undertaken to determine whether isolates might differ with respect to the carbon and nitrogen sources essential to production, and also to determine whether a correlation exists between the capacity to produce ergot alkaloids and the ability or inability to utilize certain nutrients in growth. Such information would be useful not only in understanding the biology of *Claviceps purpurea* and in improving yields, but also in designing a screening procedure for the selection of alkaloid producers from among either fresh isolates or induced mutants.

As carbon sources, glucose, sucrose, a mixture of galactose and glucose, mannitol, and lactose were selected to compare the ability of strains to produce alkaloids on various carbon sources. The galactose–glucose mixture, mannitol, and sucrose were selected because they have each been found suitable in various laboratories (1, 2, 6, 11) and glucose and lactose were selected because they are contaminants of the commercial galactose used in this laboratory (11), which led to the discovery that the mixture could be a superior carbon source (11). Ammonium succinate, yeast extract, and L-asparagine were used as nitrogen sources since they have been already tested previously (11) for one strain. Ammonium and asparagine nitrogen have also been used by other workers (1, 6, 14).

Glucose, mannitol, pure galactose, and maltose were selected as carbon sources to compare the nutritional limitations of the isolates. A direct comparison of three strains made in this laboratory (9) in 1956 failed to show

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differences in the utilization of sugars as carbon sources for growth. These results (9) did not agree, however, with nutritional studies reported by other workers (1, 4, 5, 15). While a direct comparison of the strains used in the different laboratories has not been made, a study of the effect of inoculum size on utilization of mannitol by one of our strains (7) suggested that the differences observed in the various laboratories might have been due to the use of inocula of different sizes and histories, rather than to the existence of nutritionally distinct strains. Through another and direct comparison of several strains, when using comparable inocula, it was hoped that sufficient evidence could be obtained to determine whether strain differences in utilization of carbon sources for growth do in fact exist in detectable frequencies. Succinic acid was also tested as a carbon source for growth since it was previously established that this nutrient, which stimulated alkaloid production by PRL 1578, was not only available for growth, but was, in fact, also utilized in preference to the mixture of galactose and glucose during the early period of growth (12).

Strain PRL 1578 cannot utilize L-tryptophane as a nitrogen source for growth, yet this amino acid stimulates production (11) and tracer studies have shown that it is incorporated into the ergot alkaloids (13). Since inability to utilize tryptophane for growth might be a requisite for production of the ergot alkaloids, and could, therefore, be used as a screening criterion, several isolates differing in capacity to produce ergot alkaloids were examined for their ability to utilize tryptophane in growth and to oxidize and decarboxylate tryptophane.

Another fungus, *Penicillium roqueforti* PRL 1463, was included in the study because it was found to produce substances giving a positive Ehrlich reaction (11). If it should possess nutritional properties in common with the alkaloid-producing strains of *Claviceps purpurea*, this fact would provide a basis for relating certain aspects of nutrition to ergot alkaloid biosynthesis.

Materials and Methods

Cultures

Cultures obtained from various geographical regions were selected for this study (Table I). With the exception of PRL 1555, which is derived from high-alkaloid-producing strains from Spain, none of the strains had been examined for alkaloid production prior to their receipt in this laboratory.

Inoculum

Cultures to be analyzed for alkaloids were started from approximately 5 milligrams dry weight equivalent of washed mycelium which was prepared as described previously (11). Cultures grown on plates of agar media for growth estimations were started from a small quantity of washed mycelium (ca. 0.1 mg dry weight equivalent per petri plate) to avoid stimulation attributable to the presence of nutrients in larger quantities of mycelium (7).

Media

Liquid media to be used for the production of ergot alkaloids were based on basal medium No. 10-B (11).

TABLE I

History of cultures of *Claviceps* spp. used in the study

PRL No.	Source	Region	Host
1578	W. P. Campbell	Alberta	*Phleum pratense*
1564	W. P. Campbell	British Columbia	*Bromus ciliatus*
1570	W. P. Campbell	British Columbia	*Agropyron dasystachyum*
1583	W. P. Campbell	Alberta	*Calamovilfa longifolia*
1555	Centraal Bureau voor Schimmelcultures	Spain	*Secale cereale krebs*
1565	W. P. Campbell	Alberta	*Glyceria borealis*
1715*	W. P. Campbell	Alberta	Unknown
1716†	W. P. Campbell	Alberta	Wild rice
1556	Centraal Bureau voor Schimmelcultures	Portugal	*Secale cereale*
1585	W. P. Campbell	British Columbia	*Bromus ciliatus*
1596	W. P. Campbell	Saskatchewan	*Calamagrostis inexpansa*
1595	W. P. Campbell	Alberta	*Avena fatua*
1580	W. P. Campbell	Alberta	*Bromus inermis*
1557	Authors	Saskatchewan	*Secale cereale*
1574	W. P. Campbell	Ontario	*Lolium perenne*
1734	From sclerotia donated by Eli Lilly Company	Midwest state	Unknown
1735	From sclerotia donated by Eli Lilly Company	Europe	Unknown
1566	W. P. Campbell	Alberta	*Agropyron albicans*

**Claviceps maximensis.*
†Claviceps sp.

The carbon sources were: Fisher reagent sucrose and anhydrous dextrose (glucose), Baker and Adams mannitol, NBC beta lactose U.S.P., and Difco galactose. The latter contained glucose and lactose and it was previously found (11) that growth and alkaloid production were due to the presence of glucose but not lactose. Therefore, this carbon source is referred to in this paper as a mixture of galactose and glucose. Liquid media used to test for alkaloid production on various nitrogen sources contained approximately the same quantity of succinic acid and they were adjusted to pH 5.2 with either hydrochloric acid or potassium hydroxide.

Solid media used to test for growth were solidified with 1.5% Difco Bacto Agar. The medium containing L-tryptophane as a carbon source was prepared by mixing a solution of filter-sterilized tryptophane, adjusted to pH 3 with HCl, with No. 10-B basal medium but containing ammonium phosphate as the nitrogen source. While still molten, 20 ml of this medium was poured into petri plates, each containing 500 mg sterile, dry $CaCO_3$. The final concentration of tryptophane was 1% and the final pH was 6.4. The medium containing L-tryptophane as the nitrogen source was prepared by replacing the ammonium succinate of medium No. 10-B with filter-sterilized tryptophane. The agar media used to test for growth on various carbon sources were prepared by replacing commercial galactose with the indicated substance. The carbon sources were: Fisher reagent anhydrous dextrose, purified Difco galactose, Baker and Adams mannitol, Matheson, Coleman, and Bell maltose, Eastman L-tryptophane and analar succinic acid.

Oxidation and Decarboxylation

Preparation of substrates.—Conventional Warburg techniques (16) were used and the regime was as described previously (8). Oxidation and decarboxylation of L-tryptophane were tested on 50 μmoles of tryptophane. The solution used for oxidation determinations contained sufficient glucose to give 1 μmole of glucose per vessel. The control also contained glucose. The tryptophane solutions were prepared by suspending L-tryptophane in distilled water and then adjusting the pH to 3 with HCl. Solutions adjusted to pH 10.5 with KOH were also used but since these solutions began to color within hours and therefore were constantly changing, the results obtained from their use are not reliable and are therefore not reported. Succinic and malonic acids were dissolved in distilled water, and glutamic acid was suspended in distilled water, warmed, and adjusted to pH 7–7.1 with KOH, giving a final concentration of 50 μmoles per vessel. The glutamate solution contained sufficient glucose to give a final concentration of 1 μmole per vessel. All solutions were prepared on the day they were to be used.

Preparation of mycelium.—Mycelium was obtained from 3-day-old secondary shaken cultures (11) grown on a medium consisting of 1% glucose and 1% Difco yeast extract (YD broth). Twenty hours before beginning an experiment the mycelium was blended 3 seconds and the culture returned to the shaker. Twelve hours before use, the mycelium was washed three times with sterile distilled water and suspended in *M*/15 phosphate buffer, pH 6.9. It was returned to the shaker until just before use in the respirometer. At this time the mycelium was removed by centrifugation and washed three times with phosphate–HCl buffer (8), pH 4. Finally the mycelium was suspended in this buffer to give a final concentration equivalent to 7 to 10 mg dry weight per milliliter. One milliliter was added to each vessel.

Methods used for estimation of alkaloids and other analyses have been described in earlier publications (7, 8, 9, 10, 11, 12, and 17). Cultures were incubated at $23 \pm 2°$ C.

Results

Alkaloid Production Using Various Carbon Sources

The amounts of ergot and/or clavine (14) alkaloids produced by 14 cultures on the five sugars are given in Table II. Not only do isolates differ with respect to the carbon sources which permit optimal alkaloid synthesis, but they also differ in the number of carbon sources on which they can synthesize detectable quantities of alkaloid. Glucose served as a carbon source for synthesis for 5 isolates, sucrose for 8, the mixture of galactose and glucose for 10, lactose for 1, and mannitol for 11.

One culture produced alkaloid on each of the five carbon sources; three produced on glucose, sucrose, mannitol, and the mixture of galactose and glucose; and two on the mixture and on mannitol. Two cultures produced alkaloid on mannitol only; two on sucrose, on the mixture, and on mannitol; one on sucrose and on mannitol; one on the mixture only; and one on glucose,

sucrose, and the mixture. Strain differences certainly exist, and although mannitol was not always the superior carbon source, it permitted detection of production in 11 of 13 cultures.

TABLE II

Effect of carbon source on production of alkaloids by strains of *Claviceps purpurea*

PRL No.	Day of maximal yield*	Mg alkaloid per liter				
		Glucose	Sucrose	Galactose + glucose	Lactose	Mannitol
1578	35	0.9±0.1‡	1.8±0.2	48.1±1.7	Nil	21.2±0.4
1596	45	Nil	Nil	Nil	Nil	1.1±0.1
1566	45	0.3±0.1	0.5±0.1	0.3±0.1	Nil	Nil
1565	45	10.1±3.5	13.7±1.6	3.1±0.2	4.2±0.3	9.5±1.2
1583	45	Nil	Nil	0.3±0.1	Nil	1.0±0.1
1570	45	4.3±0.5	8.4±1.2	21.2±2.6	Nil	5.5±1.3
1564	25	Nil	Nil	Nil	Nil	0.7±0.1
1585	—	Nil	Nil	Nil	Nil	Nil
1715	25	Nil	Nil	3.8±0.1	Nil	3.0±0.5
1716	25	Nil	Nil	1.4±0.1	Nil	Nil
1555	45	Nil	0.8±0.2	Nil	Nil	0.7±0.1
1735†	35	0.3±0.1	0.4±0.1	0.4±0.1	Nil	0.3±0.1
1734	35	Nil	4.6±0.2	0.5±0.1	Nil	0.3±0.1
1574	35	Nil	0.3±0.1	1.2±0.2	Nil	1.3±0.2

*Each culture was tested at 25, 35, and 45 days, and the yield is expressed as the arithmetic mean and standard error of three replicate cultures.
†The color test was blue-green, not blue.
‡The smallest standard error listed is 0.1.

Alkaloid Production Using Various Nitrogen Sources

Five cultures were compared for their ability to synthesize alkaloids on three nitrogen sources: ammonium succinate, yeast extract, and L-asparagine (Table III). Two cultures produced alkaloid on ammonium and yeast extract nitrogen and two on yeast extract nitrogen but not ammonium nitrogen. One culture did not produce alkaloid on any nitrogen source and none could utilize asparagine for synthesis.

The data given in Table III illustrate again that strain differences exist with respect to alkaloid synthesis and it is evident, when considering the data of Tables II and III, that a medium compounded for production by one strain cannot be assumed to be satisfactory for production by another strain.

TABLE III

Production on different nitrogen sources
(35 days; galactose + glucose carbon source)

PRL No.	Mg alkaloid per liter		
	Ammonium succinate	Asparagine	Yeast extract
1578	97.2±4.0	0	Trace
1570	0	0	1.4±0.3
1566	0	0	0
1565	4.8±0.6	0	49.1±2.5
1596	0	0	1.0±0.1

Comparative Growth on Carbon Sources

Sugars.—Each isolate utilized glucose and mannitol for growth but only 7 grew on galactose and 14 on maltose (Table IV). Galactose was not readily utilized by any strain. Our standard production strain, PRL 1578, which produces the highest yield on a mixture of 95% galactose and 5% glucose (11) did not grow at all on pure galactose. The data of Table IV show, however, that inability to grow on pure galactose is not associated with the ability to produce ergot alkaloids. The data given in Table IV also show that nutritionally different strains exist within *Claviceps purpurea*. Utilization of these carbon sources cannot be used to distinguish *C. purpurea* from two other species, *C. maximensis* and *Claviceps* sp. PRL 1716.

TABLE IV

Comparative utilization of various carbon sources*
(15 days)

Strain	Alkaloid produced†	Glucose	Galactose	Maltose	Mannitol	L-Tryptophane	Succinic acid
1578	+	+++	0	++	+++	0	+++
1556	−	+++	0	+	+	0	++
1564	+(−)	+++	0	++	+	0	++
1557	+	+++	+	+	++	0	+++
1570	+	+++	+	++	+	0	+
1583	+	+++	++	0	++	0	+++
1555	+	+++	0	+	++	0	+++
1565	+	+++	0	++	++	0	+++
1715‡	+	+++	++	++	+++	0	+
1716‡	+	+++	0	++	++	0	++
1585	−	+++	+	0	++	0	+
1596	+(−)	+++	+	0	++	0	+
1595	−	+++	+	+	++	0	+++
1580	−	+++	0	++	+	0	+
1574	+	+++	0	+	+	0	+++
1734	+	+++	0	+	+	0	+++
1735	+	+++	0	+	+	0	++
1463 (*P. roqueforti*)	+(?)	+++	+++	+++	+++	++	+++

NOTE: Plates of agar devoid of added carbon source were inoculated with each strain and only growth on experimental plates exceeding this quantity was considered as due to utilization of the carbon source.
*Growth estimated by visual inspection using colony diameter, thickness, and quantity of aerial hyphae as criteria of growth. Three plus signs refers to maximal growth of the indicated strain during the experiment.
†The negative sign indicates that there is no evidence for the production of alkaloid (Table II and ref. 11).
‡Different species of *Claviceps*.

Penicillium roqueforti grew on all carbon sources indicating that lack of growth by other isolates was not due to an error in the preparation of media.

L-*Tryptophane.*—L-Tryptophane stimulates production (Fig. 1) of the ergot alkaloids and increases the quantity of clavine alkaloids (11, 13). β-C^{14}-labelled tryptophane is a precursor in the biogenesis of the ergot alkaloids in PRL 1578 (13) even though tryptophane cannot be used as a sole carbon or nitrogen source for growth. This amino acid has no pronounced influence on growth or on utilization of other carbon and nitrogen sources, but does increase the total yield of alkaloid (11) (Figs. 1 and 2) and suppresses the release of organic nitrogen. The production of alkaloids may be related to the inability to use L-tryptophane as carbon or nitrogen source for growth.

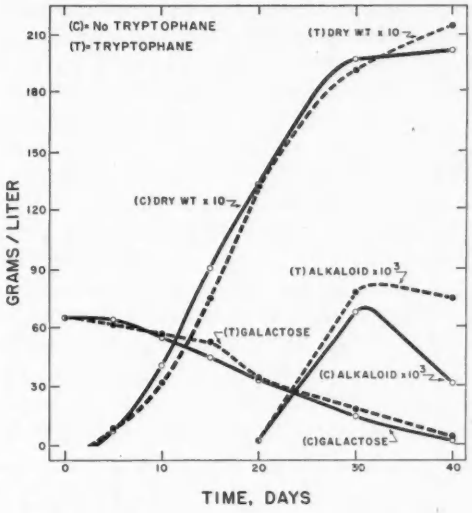

FIG. 1. Growth, utilization of the carbon source, and alkaloid production in the presence and absence of L-tryptophane by PRL 1578. Figures 1 and 2 portray data of the same experiment.

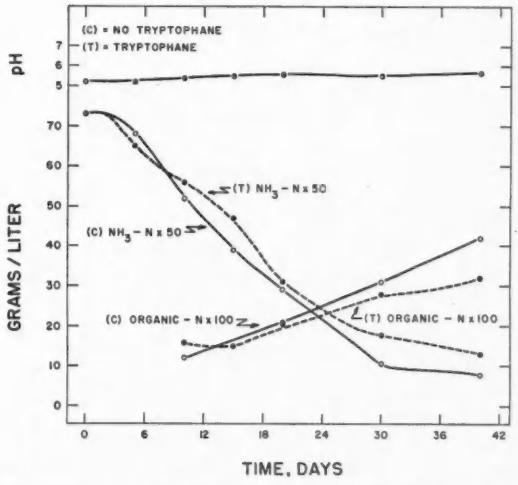

FIG. 2. Utilization of ammonium nitrogen, release of organic nitrogen, and changes in pH in the presence and absence of L-tryptophane. Figures 1 and 2 portray data of the same experiment.

However, neither producing nor non-producing isolates tested could use this amino acid as a carbon source (Table IV) and a similar survey (data not given) of these strains showed that none could use tryptophane as a nitrogen source for growth. *C. maximensis* and *Claviceps* sp. (PRL 1716) managed slight growth on a medium containing tryptophane as the sole nitrogen source. Failure to utilize tryptophane, then, is not a requisite for alkaloid synthesis. *P. roqueforti* PRL 1463 could use L-tryptophane in both capacities though to only a limited extent. Isolates PRL 1578, 1715, and 1716 were examined, in the Warburg respirometer, for ability to oxidize and decarboxylate L-tryptophane and results with PRL 1578 are given in Fig. 3. Under conditions allowing oxidation of glutamic acid, L-tryptophane was not oxidized by any of the three strains. The direct method of measurement did not reveal decarboxylation of tryptophane. Gröger *et al.* (3), however, found that tryptophane labelled with C^{14} in the carboxyl group was not incorporated into ergot alkaloids by *C. purpurea*, indicating that decarboxylation of tryptophane probably takes place during biosynthesis of the ergoline ring. Loss of the carboxyl group

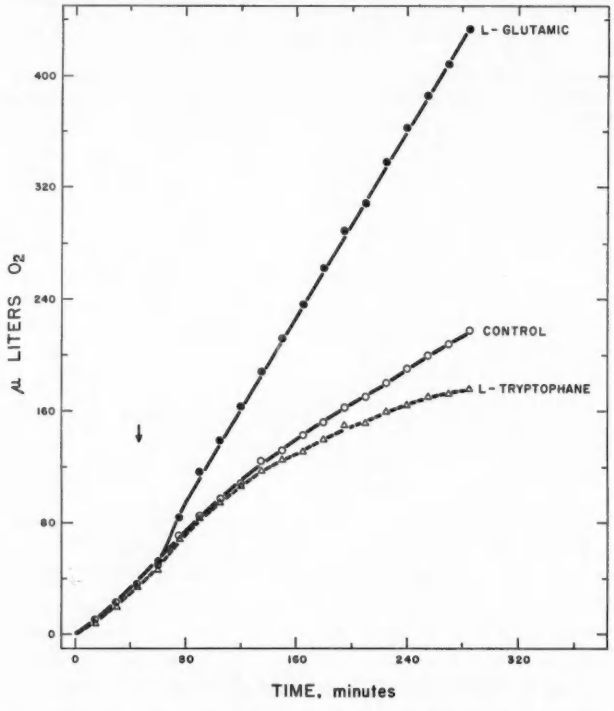

FIG. 3. Failure of PRL 1578 to oxidize L-tryptophane under conditions suitable for oxidation of glutamic acid. The side arm contained 50 μmoles amino acid + 1 μmole glucose. The control contained 1 μmole glucose. Each vessel contained the equivalent of 7.3 mg dry weight of mycelium. The pH of each vessel at the end of the experiment was 6.4.

may therefore occur only by a concurrent reaction involving condensation of a second molecule, e.g. mevalonic acid and pyrophosphate, with the activated α-carbon.

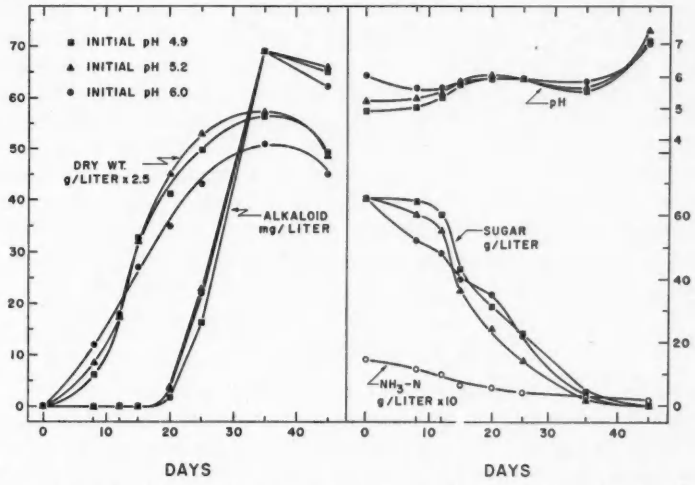

FIG. 4. Growth, utilization of sugar and ammonium nitrogen, and production of ergot alkaloids in the presence of three concentrations of succinic acid by PRL 1578. Succinic acid was added to each lot of medium to bring it to the indicated pH. The ammonium nitrogen value is the average for the three sets of cultures.

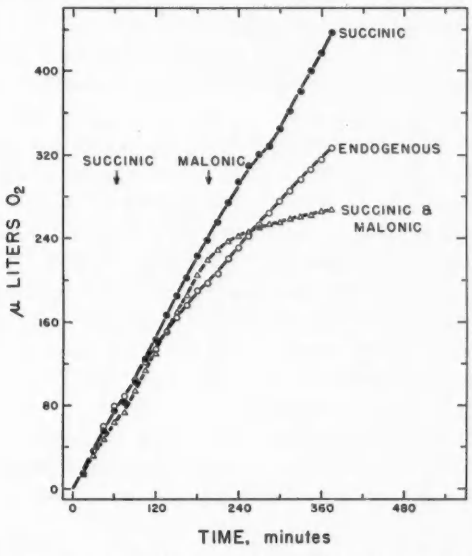

FIG. 5. Oxidation of succinic acid by PRL 1578 and inhibition by 1×10^{-2} M malonic acid. Each vessel contained the equivalent of 11 mg dry weight mycelium.

Succinic acid.—PRL 1578 produced maximum yields of alkaloid when succinic acid was present in the medium (11). It has now been shown that the specific quantity originally used is not required (Fig. 4). The role of succinic acid has not been determined but it is known that it can be used as a carbon source for growth (Fig. 4) (12) and that it spares the carbon source during the early phase of growth. Either the high concentration of succinic acid, or the low pH caused by it, however, retards this early growth (Fig. 4). Succinic acid is slowly oxidized and oxidation, including endogenous, is inhibited by 1×10^{-2} *M* malonic acid (Fig. 5). All of these data illustrate the fact that succinic acid is metabolized by a producing culture and the possibility was considered that the ability to produce ergot alkaloids was associated with the ability to metabolize succinic acid. However, when producing and non-producing strains were tested for ability to use succinic acid as a carbon source (Table IV), all were found capable of using it.

Discussion

This study has demonstrated that strain differences exist with respect to utilization of carbon and nitrogen sources for the production of ergot alkaloids. While some differences in utilization of carbon sources for growth exist, there is no correlation between utilization of any of them for growth and the capacity to synthesize ergot alkaloids. The comparative utilization of two substances involved in the biogenesis of ergot alkaloids, succinic acid and tryptophane, was examined. Both producing and non-producing strains could use succinic acid as a carbon source for growth and none could use L-tryptophane as a carbon or nitrogen source for growth.

The ability to synthesize ergot or clavine alkaloids is not limited to *C. purpurea*. Two other species, *C. maximensis* and *Claviceps* sp. PRL 1716* synthesized alkaloids.

Mannitol would be the best single carbon source of those tested to use in a screening procedure since most strains produced detectable quantities of alkaloid on it. The fact that strains often produced alkaloid on more than one carbon source and that they differed as to the "optimal" carbon source for production indicates, however, that a medium which allows maximum production for one strain may not be satisfactory for production by another strain. The nitrogen source is also an important variable, and, although ammonium nitrogen appears to be widely used for production, a second nitrogen source, yeast extract, included in a screen might result in the detection of additional isolates capable of production.

The fungus *Penicillium roqueforti* PRL 1463, which produces substances giving a positive reaction with *p*-dimethylaminobenzaldehyde, bears no strict nutritional similarities to alkaloid-producing strains of *Claviceps purpurea*.

*These species were obtained from and identified by Dr. W. P. Campbell, Plant Pathology Laboratory, Canada Department of Agriculture Research Branch, Edmonton, Alberta.

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THE CULTURE OF DUNALIELLA TERTIOLECTA BUTCHER—A EURYHALINE ORGANISM[1]

JACK MCLACHLAN[2]

Abstract

Dunaliella tertiolecta Butcher was found to be a euryhaline organism which grew at salinities ranging from 3.75 to 120°/$_{oo}$. All the conservative elements of sea water, with the exception of chlorine, were found necessary for growth of the alga. The minimum requirement for sodium was much greater than that for any other element, and it was not possible to substitute other monovalent cations for the minimum requirement. Also, the alga could tolerate high concentrations of sodium chloride. The potassium and sulphur concentrations of the medium could be reduced to very low levels. *Dunaliella* could also tolerate high concentrations of these two elements. The addition of lithium to the medium inhibited the growth of the alga. High concentrations of sodium could partially eliminate the inhibition due to lithium. The minimum concentrations of calcium and magnesium necessary for growth approached the concentrations found in fresh waters. Calcium and magnesium were inhibitory at high concentrations, but the inhibition at high concentrations could be prevented if a Mg/Ca ratio of 4 was maintained over a wide range of concentrations in the medium.

Introduction

The genus *Dunaliella* includes both fresh and salt water species (22), a situation which is not uncommon (9). This genus is of world-wide distribution and is generally present in brine lakes (4, 15, 29). *Dunaliella* has been observed to bloom under conditions of high salt concentration (20), and some strains are able to grow in media saturated with sodium chloride (14, 29).

Some species of *Dunaliella*, including *D. tertiolecta*, occur in sea water (cf. ref. 8). As a medium, sea water is a complex solution of salts, and final analysis may reveal the presence of all elements of the periodic table (21). The conservative elements[3] in sea water are present in large concentration and occur at a constant ratio regardless of the salinity of the water. Most of these elements are known to be essential constituents in plant nutrition. Kalle (21) considers sea water as an ideal medium for growth of marine plants, but very little is known of the relationship between the concentration of conservative elements and growth of microorganisms (10, 38). The few studies which have been made (1, 10, 17, 33, 34) have indicated considerable liberty can be taken with the composition of the medium. A number of fresh-water algae have been found to grow well and tolerate conditions of high salt concentration (13, 36). It has been suggested that, as a group, green algae seem to be most adaptable to a wide variety of conditions (32).

Previous work with *Dunaliella tertiolecta* has shown that the alga could be cultured equally well in an artificial medium and in an enriched sea water

[1]Manuscript received February 24, 1960.
Contribution No. 1097 from the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, U.S.A.

[2]Public Health Service Research Fellow of the National Institutes of Health. Present address: Division of Applied Biology, National Research Council, Ottawa, Canada.

[3]Conservative elements as used in this paper refer to Cl^-, $SO_4^=$, Na^+, Mg^{++}, Ca^{++}, and K^+. In sea water the ratio of these ions remains virtually unchanged regardless of the salinity, or through the activity of plants and animals.

medium (24). It was the purpose of this study to investigate the concentration limits of the various conservative elements of sea water which are required for growth or are tolerated by *D. tertiolecta*, and to determine if the alga had any requirements which would clearly distinguish it as a marine organism.

Materials and Methods

An axenic culture of *Dunaliella tertiolecta* Butcher[4] was used throughout this investigation. The alga had been originally isolated from sea water but was cultivated on an artificial medium for approximately a year prior to the experimental work. The basic artificial medium, which contained no organic growth factors, was the same as used in previous work (24). The composition of this medium (designated A medium) and of two modifications which were also used (designated B medium and C medium) are presented in Table I. In B medium only the sodium chloride concentration was reduced; in C medium the concentrations of sodium chloride, magnesium sulphate, magnesium chloride, and calcium chloride were reduced as compared with A medium.

TABLE I

Composition of the basic artificial media*

A medium			
$NaCl$	410 mM	$FeCl_3$	1.5 μM
$MgSO_4$	24 mM	H_3BO_3	185.0 μM
$MgCl_2$	20 mM	$MnCl_2$	7.0 μM
$CaCl_2$	10 mM	$ZnCl_2$	0.8 μM
KNO_3	1 mM	$CoCl_2$	0.02 μM
K_2HPO_4	100 μM	$CuCl_2$	0.0002 μM
Na_2SiO_3	100 μM	Na_2EDTA	30.0 μM
B medium			
$NaCl$	15 mM		
Other salts as in A medium			
C medium			
$NaCl$	50.0 mM	$MgCl_2$	2.75 mM
$MgSO_4$	2.0 mM	$CaCl_2$	1.25 mM
Other salts as in A medium			

*Chlorides standardized by titration.

The alga was cultivated in 125-ml Erlenmeyer flasks containing 50 ml of medium. The flasks were plugged with cotton and were shaken by hand several times daily. Illumination was provided by cool-white 40-w fluorescent lamps with an intensity at the level of the culture shelf of approximately 3500 meter-candles, as measured by a General Electric type DW-68 light meter. The temperature was maintained at 18° C.

Cells were harvested from either two or three flasks for each treatment within an experiment. The cells were killed and stained with $K_2 - I_2 -$ acetic

[4]Previously this strain was referred to as *D. euchlora* WHOI-1 (24, 25, 26), but recently was identified correctly by Dr. R. W. Butcher as *D. tertiolecta*. This type was first isolated from Oslo Fjord (8).

acid. The rate of growth was determined periodically by making four to eight replicate cell counts per treatment using a Levy hemocytometer counting chamber. The most rapid rate of growth in each treatment is reported. The cultures were harvested in the log phase of growth; the concentration of cells at this time was usually between 100×10^4 and 200×10^4 cells per milliliter. The maximum cell concentrations obtained under the experimental conditions were approximately 5×10^6 cells per milliliter. The rate of growth is expressed as the number of divisions per day using the equation: $\log_2 (N_t/N_0)/t$ where N_0 is the concentration of cells at inoculation and N_t the concentration of cells at time t (27).

Results

1. Salinity

The concentrations of sodium chloride, magnesium chloride, magnesium sulphate, and calcium chloride were varied from 1/16× to 4× the concentration of A medium. Concentrations greater than 4× that of A medium precipitated and were therefore not used. The rates of growth from 1/2× to 2× were approximately the same (Fig. 1). The rates of growth at concentrations of 1/8× and 4× were considerably reduced, but these treatments ultimately achieved the same final cell concentration as the treatments at concentrations from 1/4× to 2×. No growth occurred at 1/16× concentration. Growth occurred over a range of salinities from 3.75 to 120‰. These results suggest that the rate of growth may have been limited by high or low total salt concentration of the medium.

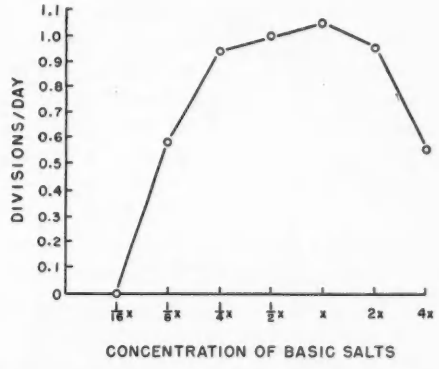


FIG. 1. The rate of growth of the alga at various salinities. × represents the concentration of basic salts (salinity) in A medium.

2. Sodium Chloride

Preliminary results showed that the concentration of sodium chloride in A medium could be varied from 25 to 1000 m*M* without affecting the rate of growth or the final cell concentration.

In A medium containing more than 1000 m*M* sodium chloride, the rate of growth was reduced, and growth was completely suppressed at 2000 m*M* (Fig. 2). However, in all treatments in which growth occurred, the same final cell concentration was obtained. The rate of growth in the 1800 m*M* sodium chloride treatment was approximately the same as the rate of growth of the 4× treatment of the previous experiment (Fig. 1). The total salt concentration of the 1800 m*M* sodium chloride and 4× treatments was approximately the same (ionic concentration ca. 3800 m*M*), which indicates the osmotic pressure between 1000 and 1800 m*M* sodium chloride affected the rates of growth. The sharp break in the response curve between 1800 and 2000 m*M* sodium chloride may suggest that sodium chloride is intrinsically toxic at higher concentrations.

The treatments containing 0 to 25 m*M* sodium chloride were inoculated from a stock culture grown on B medium, so very little sodium chloride was carried over with the inoculum. The rates of growth decreased from 25 to 10 m*M* sodium chloride, and more rapidly from 10 to 0 m*M* sodium chloride (Fig. 2). These results suggest that the decrease in the total salt concentration between 25 and 10 m*M* sodium chloride affected the rate of growth. The rate of growth between 0 and 10 m*M* was also affected by the decreased salt concentration of these treatments. However, the total salt concentration in treatments from 0 to 10 m*M* sodium chloride exceeded the total salt concentration of the 1/8× treatment of the previous experiment. As none of the treatments from 0 to 8 m*M* sodium chloride obtained a maximum cell concentration, these results suggest that sodium chloride per se limited growth.

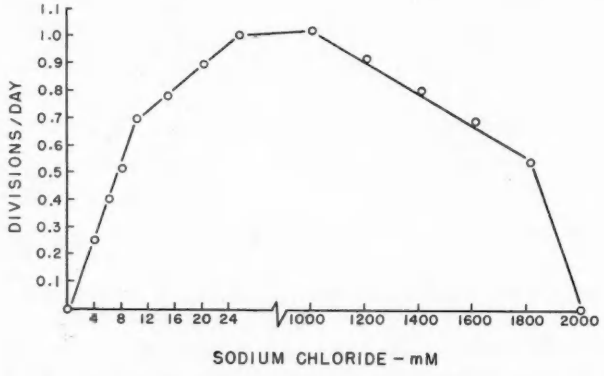


FIG. 2. The rate of growth of the alga in various concentrations of sodium chloride. The concentration of the other salts was maintained at the concentration of A medium.

The rate of growth in C medium was unaffected between 80 and 100 m*M* sodium chloride (Fig. 3). A decrease in the rate of growth occurred in all treatments containing less than 80 m*M* sodium chloride, but the maximum cell concentrations were obtained in all treatments in which growth occurred.

In the lowest sodium chloride concentration in which growth occurred (30 mM), the total salt concentration was considerably less than that of the 0 mM sodium chloride treatment of the previous experiment. Therefore, growth in this experiment was suppressed by a low total salt concentration before sodium chloride became deficient.

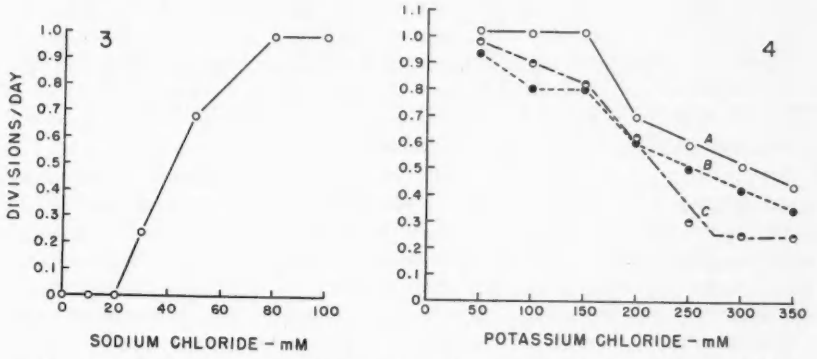


FIG. 3. The rate of growth of the alga in various concentrations of sodium chloride. The concentration of the other salts was maintained at the concentration of C medium.
FIG. 4. The rate of growth of the alga in various concentrations of potassium chloride in A, B, and C media.

3. Potassium Chloride

The addition of 50 mM potassium chloride to B and C media increased the rate of growth over that of the controls containing 1.2 mM potassium (as potassium nitrate and potassium phosphate). Concentrations exceeding 50 mM potassium chloride depressed the rate of growth (Fig. 4). The rate of growth in A medium was unaffected until the potassium chloride concentration exceeded 150 mM. These results suggest a slight antagonism between potassium and sodium between 50 and 150 mM potassium as B and C media contained considerably less sodium than A medium. The inhibition in all cultures containing more than 150 mM potassium chloride was probably due to potassium toxicity per se.

Stock cultures in A medium were grown without the addition of potassium; nitrate and phosphate were added as sodium salts. The stock cultures were allowed to grow for 12 days and then inoculated into media containing micromolar concentrations of potassium chloride. The results (Fig. 5) show that 40 to 50 μM potassium were necessary to permit a normal rate of growth. Essentially the same final cell concentration was obtained in treatments containing 30 μM or more potassium. Cell division could be directly related to the concentration of potassium in the medium (Table II). From these data it was possible to calculate that the alga required approximately 6.0×10^{-8} μg-atoms of potassium per cell. These results also show that sodium could not be substituted for potassium in growth of the alga.

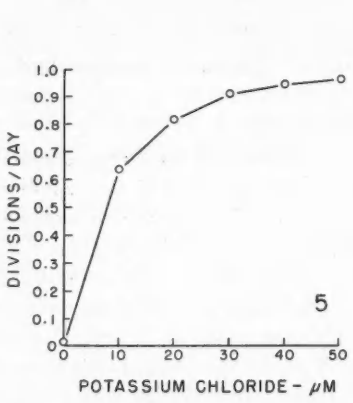
TABLE II

The effect of various concentrations of potassium on the maximum cell concentration

μM potassium added	No. cells/ml $\times 10^4$	Per cent of control	μg-atoms potassium per cell $\times 10^{-8}$
0	No growth	—	—
10	168	32	5.95
20	337	64	5.94
30	495	94	6.04
40	485	93	—
50	510	97	—
1200	525	100	—

4. Sodium Chloride and Potassium Chloride

Preliminary results indicated that it was not possible to substitute potassium chloride for sodium chloride. Two series of cultures were established with the concentration of sodium chloride varied in each series from 0 to 25 mM; magnesium and calcium salts were added at the concentration of B medium. In one series potassium chloride was added at the concentration of B medium (1.2 mM), and in the other series at a concentration of 50 mM.



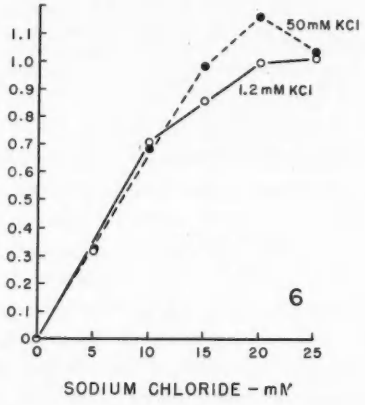


FIG. 5. The rate of growth of the alga in various micromolar concentrations of potassium chloride in A medium.

FIG. 6. The rate of growth of the alga in various concentrations of sodium chloride in B medium to which either 50 mM or 1.2 mM potassium chloride was added.

All cultures were inoculated from B medium stock cultures to reduce the amount of sodium chloride carried over with the inoculum. No increase in the rate of growth was effected by the addition of 50 mM potassium chloride in cultures containing 10 mM or less sodium chloride (Fig. 6), even though the total salt concentration of the medium was sufficient to permit a more rapid rate of growth. The rate of growth was, therefore, limited by the sodium chloride concentration. Fifty millimolar potassium chloride stimulated the rate of growth at sodium chloride concentrations of 15 and 20 mM. The rate of growth in the 20 NaCl – 50 KCl treatment was greater than that of the other

treatments, and was the most rapid rate recorded during this study. The rates of growth in the 15 NaCl – 50 KCl and 25 NaCl – 50 KCl treatments were comparable with the highest rates obtained in other experiments in this study.

5. Lithium Chloride

All concentrations of lithium chloride added to A, B, and C media inhibited the rate of growth (Fig. 7). A maximum cell concentration was obtained only in A medium containing 50 m*M* lithium chloride. These results suggested an antagonism between sodium and lithium as A medium contains considerably more sodium than B and C media. Cells of treatments in which maximum cell concentrations were not obtained became greatly enlarged, and after a short period of growth, chlorotic.

In treatments containing 50 m*M* lithium chloride and various concentrations of sodium chloride, the rate of growth was dependent upon the sodium chloride concentration (Fig. 8). However, concentrations of sodium chloride greater than 400 m*M* did very little to enhance the rate of growth. Maximum cell concentrations were obtained only in treatments containing 200 m*M* or more sodium chloride. As in the previous experiment, cells in the lower sodium chloride concentrations enlarged and soon turned chlorotic.

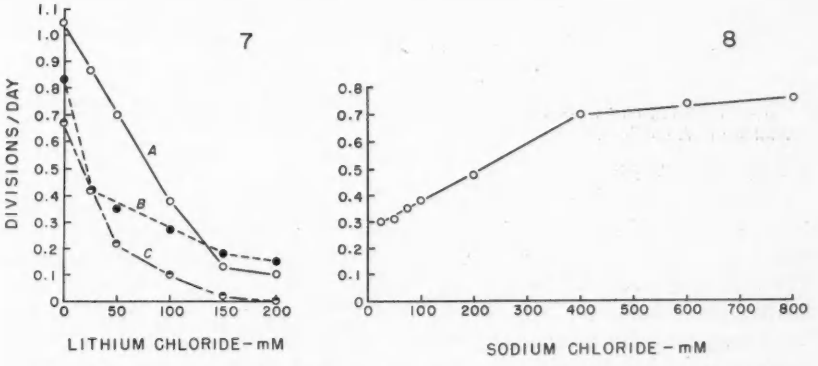


FIG. 7. The rate of growth of the alga in various concentrations of lithium chloride in A, B, and C media.

FIG. 8. The rate of growth of the alga in various concentrations of sodium chloride in B medium with all treatments containing 50 m*M* lithium chloride.

6. Sulphur

Cultures containing micromolar concentrations of sodium sulphate were inoculated from a stock culture which contained 0.25 m*M* sodium sulphate. In all treatments, magnesium was added as magnesium chloride at the concentration of A medium. No growth occurred in the treatment in which sulphur had been omitted. A minimum concentration of approximately 150 μM was necessary to effect a normal rate of growth (Fig. 9). All treatments containing 50 μM or more sodium sulphate reached the same final cell concentration.

The final cell concentration of the 25 μM treatment was only 26% of the final cell concentration of the 50 μM treatment. This may suggest that sulphur is not directly involved in cell division. Hase *et al.* (16) found a sulphur deficiency unique in causing a complete cessation of cell division.

It was possible to add sodium sulphate up to a concentration of 300 mM in A medium without affecting the rate of growth or the final cell concentration. Concentrations exceeding this resulted in a precipitation of calcium sulphate. It was also possible to replace sodium chloride in A, B, and C media with sodium sulphate without affecting growth. Some studies (20) have indicated that it is not possible to replace sodium chloride with other sodium salts.

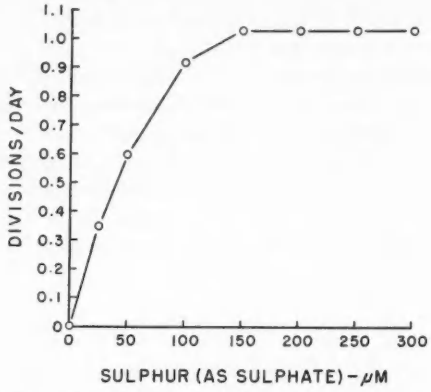


FIG. 9. The rate of growth of the alga in various micromolar concentrations of sodium sulphate in A medium.

7. Calcium Chloride

Calcium chloride was added at various concentrations to A, B, and C media. The carry-over of calcium with the inoculum was about 200 μM in treatments based on A and B media, and about 25 μM in treatments based on C medium. The rate of growth was inhibited at 50 mM in A medium, at 40 mM in C medium, and at 30 mM in B medium (Fig. 10). These results suggest that the inhibitory effects of excess calcium were antagonized by sodium since one of the important differences between B medium and C and A media is that the sodium chloride concentration is more than three to almost 30 times higher in the latter two than in the former. All treatments containing added calcium reached the same final cell concentration. The low rate of growth in A medium with low calcium concentration may also indicate an antagonism between calcium and sodium chloride. In B and C media, the reduced rate of growth at the lower calcium concentrations undoubtedly reflects the low total salt concentration of these treatments.

8. Calcium Chloride and Sodium Chloride

Treatments were prepared with 60 mM calcium chloride and concentrations of sodium chloride from 25 to 800 mM. At concentrations of 25 and 50 mM sodium chloride, the reduced rate of growth was similar to that of the previous

experiment, i.e. in the B and C media (Fig. 10). The rate of growth from 100 to 800 m*M* sodium chloride was the same and comparable with the 60 m*M* calcium chloride (A medium) of the previous experiment. Therefore, inhibition due to excess calcium can be somewhat mitigated by increasing the sodium concentration, but the inhibition of the rate of growth could not be entirely overcome by an increased sodium concentration.

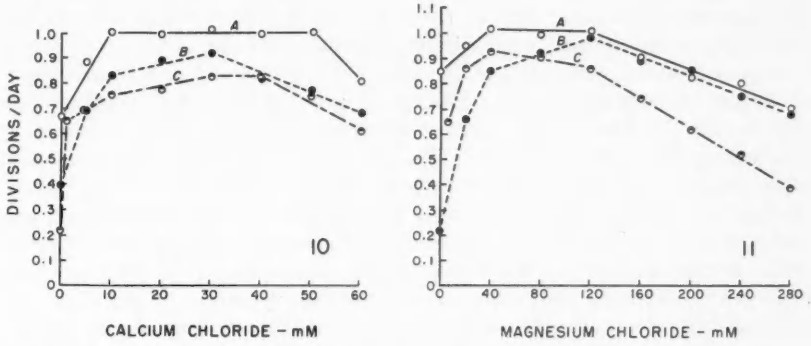


FIG. 10. The rate of growth of the alga in various concentrations of calcium chloride in A, B, and C media.

FIG. 11. The rate of growth of the alga in various concentrations of magnesium chloride in A, B, and C media.

9. Magnesium Chloride

Magnesium chloride inhibited the rate of growth at concentrations exceeding 120 m*M* in A, B, and C media (Fig. 11). The degree of inhibition of the rate of growth may be related to the Mg/Ca ratio as C medium contains considerably less calcium than A and B media. All treatments based on A medium and all treatments based on B medium, except the one containing no added magnesium, reached the same final cell concentration. A maximum cell concentration was not obtained in treatments containing 240 and 280 m*M* magnesium chloride in C medium. This again suggests inhibition due to excess magnesium which was less antagonized by calcium.

10. Calcium and Magnesium

Treatments were established in which the Mg/Ca ratio was held constant at 4.0, but the concentration of Mg/Ca was varied from 0.8/0.2 to 240/60 m*M*. There was no difference in the rate of growth over this range of magnesium and calcium concentrations, and all cultures reached the same final cell concentration. Therefore, inhibitory effects of excess magnesium and calcium could be overcome by maintaining a favorable Mg/Ca ratio.

Discussion

The results of this investigation have shown that *Dunaliella tertiolecta* is definitely an euryhaline organism. Other studies with this genus (14, 22) and with other organisms (cf. refs. 6, 7, 35) have demonstrated tolerances to a

wide range of salinities. Most organisms studied, though, do not grow well at salinities much greater than normal sea water (35‰). Some investigators (1, 13) were able to grow their organisms at concentrations varying from those present in fresh waters to full strength sea water. However, *Dunaliella tertiolecta* could not tolerate an osmotic pressure comparable with fresh waters.

Dunaliella tertiolecta has a conspicuous requirement for sodium. Sodium could be provided either as sodium chloride or sodium sulphate. Presumably other sodium salts could also suffice as long as the anion was not toxic. The requirement for sodium was specific, and none of the other monovalent cations examined were able to replace the minimum sodium requirement.

Osterhout (28) reported a sodium requirement in macroscopic marine algae, and several studies have confirmed a requirement in microscopic marine algae (10, 17, 18, 19, 31). In some of these studies it was shown that some organic compounds were able to substitute partially, but not completely, for sodium. Fungi (38) and bacteria (20) have a sodium requirement, and Stiles (37) has indicated a requirement for sodium may be a general phenomenon among marine plants. On the other hand, other studies have shown that not all marine algae have an obvious sodium requirement. Allen (1) and George (13) were ostensibly able to grow several marine species in sodium-free media, and Mayer (23) has found a marine *Chlorella* which grows better when sodium is omitted from the medium.

In blue-green algae, a group virtually unknown from the plankton of the sea (5), some fresh-water species have an absolute sodium requirement (2, 3). Other studies with fresh-water algae have shown a stimulation from the addition of sodium while others have shown that sodium can partially replace potassium (37).

Potassium was found necessary for the growth of *Dunaliella*, but only in quantities which presumably fulfilled the metabolic requirements of the alga. There was no indication that sodium could replace potassium as a metabolite. The quantity of potassium necessary for normal growth was far less than the concentration found in sea water. Pintner and Provasoli (31) and Vishniac (38) also demonstrated that the potassium requirement for several other marine organisms could be greatly reduced from that of sea water. At the other extreme, *D. tertiolecta* was able to tolerate a potassium concentration many times that of sea water.

Osterhout (28) demonstrated that lithium was not able to replace sodium. In the present study, it was also shown that lithium could not replace sodium in the growth of *Dunaliella*. Lithium was found to be toxic at all concentrations used. A beneficial action from the addition of lithium has been observed in the growth of some plants, but more generally the effect is depressing (37). Frerking (12) has claimed that lithium is toxic to plants requiring calcium, but not to algae and fungi supposedly free from calcium.

If chloride is necessary for growth of *Dunaliella*, it is in much less demand than sodium. It was not possible to replace all chloride with sulphates due to difficulties with precipitation. It has not been demonstrated that chloride is

necessary for the growth of marine algae, but it has been found essential in the development of some land plants (37). Eyster (11) did find that the growth of a fresh-water alga under autotrophic conditions was stimulated by the addition of chloride.

It was possible to obtain a normal cell concentration of *Dunaliella* at a calcium concentration comparable with that of fresh waters. Droop (10) and Hutner (17) have also reported calcium requirements of the same order of magnitude. Contrariwise, Allen (1) found little flexibility with regard to calcium, and reduced growth resulted when the concentration fell below that of normal sea water.

Growth of *Dunaliella* occurred over a wide range of magnesium concentrations varying in concentration from that characteristic of fresh waters to a concentration greatly exceeding that of normal sea water. Similar results were also obtained by Droop (10) and Hutner (17), but one of the organisms studied by Droop had a magnesium optimum higher than that of North Atlantic waters. Pintner and Provasoli (31) found their organisms were inhibited at a magnesium concentration greater than that of normal sea water.

Vishniac (38) and Vollenweider (39) have reported that magnesium and calcium could replace each other to a certain extent. Several experiments were carried out to determine if either element could substitute for the other in the growth of *D. tertiolecta*. All experiments were negative and indicated substitution did not occur. The alga could tolerate, however, wide variations in the Mg/Ca ratio as long as one or the other was not limiting. This also seems to be true of other organisms (32). Toxic effects of high concentrations of calcium or magnesium could be eliminated by maintaining a proper Mg/Ca ratio. Pintner and Provasoli (30) also indicated that toxic effects of high magnesium concentrations in their medium could be overcome by increasing the calcium concentration.

With the possible exception of chloride, all major elements in sea water were found necessary for the growth of *Dunaliella tertiolecta*. Apart from sodium, the concentrations necessary for the development of this alga were of the same order of magnitude as those necessary for the growth of fresh-water algae. The osmotic and sodium requirements are the principal features which distinguish this alga as a marine organism. The sodium requirement is not merely one of osmoregulation as other salts cannot substitute for sodium. Other salts can substitute for sodium as osmoregulators as long as a minimum concentration of sodium (ca. 10 mM) is maintained in the medium. It can be concluded that, when culturing this organism, the first deficiency to be encountered will be sodium. Droop (10) has reached a similar conclusion with other organisms.

The alga also showed considerable tolerance to increased concentrations of other elements in sea water. In concentrating sea water, the osmotic pressure of the medium limited growth before a single element became toxic. By increasing the concentration of each element singly, magnesium was found to be most toxic. Generally the dominant ion in alkali lakes is magnesium which

may explain the absence of this species from these habitats, particularly if the calcium concentration is low. The alga was more tolerant towards high concentrations of potassium than towards the other elements.

It was not possible to increase significantly the rate of growth over that of enriched sea water media (24) under the various conditions used in this study. This would tend to support the conclusion of Kalle (21) that sea water is an ideal medium for plant growth.

Acknowledgments

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NOTES

MEASUREMENT OF GENERATION LAG

W. R. LOCKHART

The lag time of Hinshelwood (2) may be defined as the time at which growth of a culture would commence if all cells in the inoculum began to reproduce simultaneously at the maximum logarithmic rate. But it is characteristic of lag that early population increase occurs at low rates which gradually accelerate toward the logarithmic-phase maximum. Further, when lag is measured by this method or by variations thereof (1), accurate comparisons are not possible between cultures with different rates of logarithmic growth (L and L' in Fig. 1).

Generation lag (L_g), the alternative measurement proposed here, is based on estimating the time at which is accomplished the first doubling of the inoculum population (i.e., the initial generation of growth). If base 2 logarithms of cell concentration are plotted, as in Fig. 1, L_g may be determined graphically. It exceeds lag time (L) by a period equal to the average generation time during logarithmic growth ($1/R$ in terms of Fig. 1).

Since L may be calculated as:

$$L = t_1 - \{(G_1 - G_0)/R\},$$

then

$$L_g = t_1 - \{(G_1 - G_0 - 1)/R\}.$$

If the data are to be handled in base 10 logarithms, this becomes:

$$L_g = t_1 - 3.32g\ (\log N_1 - \log N_0 - 0.30),$$

where g is generation time in hours and N_1 and N_0 are the cell counts corresponding to G_1 and G_0.

This procedure has the effect of cancelling differences in calculated lag time which derive from variations in the rates of subsequent logarithmic growth. Table I shows the results of an experiment in which growth rates were a function of oxygen availability. Although early growth is not much different in aerated and unaerated cultures, the calculated L values are not at all comparable. It would appear that L_g is a better measure of lag in this instance.

The data in Table I indicate that L_g (the time when the inoculum population has just doubled) is very near the time at which lag has actually ended; i.e., the point beyond which the population as a whole increases at the maximum logarithmic rate. Similar indications have been found in growth data obtained with various strains of *Escherichia coli* studied in this laboratory. That this would be the case is an assumption which is perhaps implied in the concept of generation lag; it will be interesting to find whether this pattern is of general occurrence in growth of other bacteria. At present, however, L_g is best regarded as a strictly arbitrary measure. It nevertheless seems justifiable

in experimental situations, like the one illustrated, where it yields results superior to those obtained with Hinshelwood's *L*. Whichever way one attempts to measure lag, he can only (as Finn so aptly stated) "resort to some derived parameter and thereby gain precision at the expense of accuracy".

TABLE I

Growth of aerated and unaerated cultures of *Escherichia coli* K12/G2 in synthetic medium

Incubation time (hours)	Log_2 total cells per ml	
	Unaerated culture	Aerated culture
0	24.3	24.3
1	24.5	24.4
2	25.1	25.0
3	25.5	25.8
4	26.1	26.8
5	26.6	27.9
6	27.0	29.0
R*	0.5	1.1
L	0.5	1.6
L_g	2.5	2.6

*R = logarithmic growth rate, generations/hr.
L = lag time, hours.
L_g = generation lag, hours.

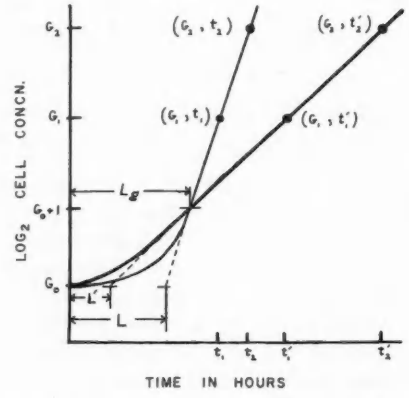

FIG. 1. Comparison of lag time and generation lag. Logarithmic growth rate (generations per hr) = $R = (G_2 - G_1)/(t_2 - t_1)$.

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A PRELIMINARY INVESTIGATION OF THE PIGMENTS OF TRICHOPHYTON RUBRUM

MALATI R. BAICHWAL[1] AND G. C. WALKER[2]

Pigments produced by different fungi have been utilized by mycologists as an aid in the identification and characterization of fungi. Dermatophytic fungi are no exception and many produce pigments. As early as 1910, Sabouraud (3) had examined the pigments of the dermatophytes and reported them to be acid in nature and precipitated by alkali. No further work was carried out on the pigments for over 15 years, after which Tate (4) examined the pigments produced by some species of *Trichophyton*. The pigments of several species (*T. megnini*, *T. sabouraudi*, *T. rubrum*, and *T. mentagrophytes*) were found to be red to reddish brown, easily soluble in dilute acids and acid alcohol, but only very slightly soluble in dilute alkali. Upon extraction, the color in acid solution was yellow and changed to red or reddish brown in alkaline solution. The color change was easily reversible and the pigment thus appeared as an indicator of hydrogen ion concentration. Tate suggested that the pigments might be of an anthracene nature, resembling the yellow pigments of the lichen *Physcia*.

Thompson (5) confirmed Tate's findings and pointed out that the pigments of *T. rubrum* and *T. mentagrophytes* were soluble in acetone. The pigment seemed to be a pH indicator with an oxidized and a reduced form.

While working with some strains of *T. mentagrophytes*, which produced red pigment, Georg and Maechling (1) reported that the pigment appeared to be anthraquinone in nature and to occur as a glycoside.

During the progress of an investigation of the pigments of *T. rubrum* made at the laboratories of the Faculty of Pharmacy, University of Toronto, Wirth *et al.* (6) reported the isolation in crystalline form of some pigments of *T. rubrum*. The crude red pigment was extracted using ethylene dichloride and fractionated by column chromatography and rechromatography into three crystalline substances and some fractions. The crystalline substances were designated as purple needles, orange plates, and red needles. They suggested that the three substances were different from each other on the basis of the following tests: solubility in 5% sodium carbonate; solubility in 5% sodium bicarbonate; colors obtained in 1 *N* sodium hydroxide, concentrated sulphuric acid, sodium dithionite solution, and glacial acetic acid. At the same time Mier (2) using ascending paper chromatography separated a crude glacial acetic acid extract into three major fractions. The pigments were believed to be derivatives of anthraquinone and an absorption spectra over the range 200–600 mμ was reported.

This paper is concerned with the results of a study relating to the isolation of pigment material produced by *T. rubrum*.

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Organism.—A strain of *T. rubrum* (No. 3491) isolated from a patient and known to produce bright-red pigment when grown on artificial media was obtained from the Central Laboratories of the Ontario Department of Health, Government of Ontario.[3]

Collection of mycelium.—One-liter conical flasks containing 200 ml of sterile Sabouraud's Dextrose Broth (peptone 10 g, dextrose 20 g, distilled water 1000 ml) were inoculated with 1 ml of a standardized conidial suspension containing approximately five million conidia per milliliter. The flasks were incubated at 27° plus or minus 2° for a period of 3 weeks. The mycelium was collected and dried at 45°. The yield of mycelium per flask was about one gram.

Extraction.—Extraction of the pigment was first attempted using common organic solvents, alkalis, acids, and distilled water. A solution of sodium hydroxide 5% appeared to extract the pigment completely leaving a colorless mycelium. Wirth *et al.* (6) observed that ethylene dichloride did not completely exhaust the mycelium no matter how prolonged the extraction period. The powdered mycelium (75 g at one time) was extracted with a solution of 5% sodium hydroxide at room temperature. About four liters of alkali were required for complete extraction. The violet to pink sodium hydroxide solution was acidified with hydrochloric acid and extracted with chloroform to give a yellowish solution. Extraction of this solution with sodium bicarbonate 5% showed a violet to pink color in solution which on further purification with successive acid and chloroform treatments and evaporation *in vacuo* gave a light-yellow, amorphous powder from chloroform. A trace of this yellow substance gave an intense red color with alkali. This yellow powder was used for the purposes reported.

Chromatography.—Preliminary trials using various columns and solvent systems were made and established the usefulness of acid alumina with ethylene dichloride containing 1% glacial acetic acid as the eluting solution. The results are shown in Table I.

TABLE I

Chromatographic data on the "bicarbonate-soluble" pigment of *T. rubrum* using acid alumina as the adsorbent

Eluant, ethylene dichloride containing acetic acid in concentration of: (per cent)	Eluate No.	Color of the band	Coloration with 5% NaOH
0	I	—	Colorless
1	II	—	Colorless
1	III	Yellowish	Light pink
2 4	IV	Light brown-violet	Light pink
8	V	Diffused brown	Pink
20[a]	VI	Brown	Pink

[a]Aqueous acetic acid.

[3]The authors wish to thank Mr. J. B. Fischer of the Mycology Section of the Ontario Department of Health, Province of Ontario, for supplying the organism used in this study.

Absorption spectra.—The ultraviolet and visible absorption maxima of a sample of the extracted pigment matter and of the elution bands from chromatographic separation are recorded in Table II. A Beckman model DU spectrophotometer was used for this purpose. The absorption curves were determined repeatedly during the investigation and the peaks were found to be in the same range. The ultraviolet absorption spectrum of the "bicarbonate-soluble" pigment (light-yellow powder) and of the pigment extracted by soxhlet for comparison purposes and by using ethylene dichloride are shown in Figs. 1 and 2.

TABLE II

Absorption spectra in ultraviolet and visible regions of the pigment material of *T. rubrum*

No.	Description of the sample of the pigment	Solvent	Absorption maxima at wave lengths (in mμ) in: Ultraviolet	Visible
	Sample 'Bicarbonate-soluble' light-yellow powder	Methanol	274 to 276	392 to 394
	Eluates*			
III	Yellow band	Methanol	272 to 274	Nil
IV	Light brown-violet band	Methanol	272 to 275	382 to 386
V	Diffused brown band	Methanol	268 to 272	384 to 390
VI	Brown band	Methanol	260 to 274	401 to 405

*Eluates—refers to the fractions as outlined in Table I.

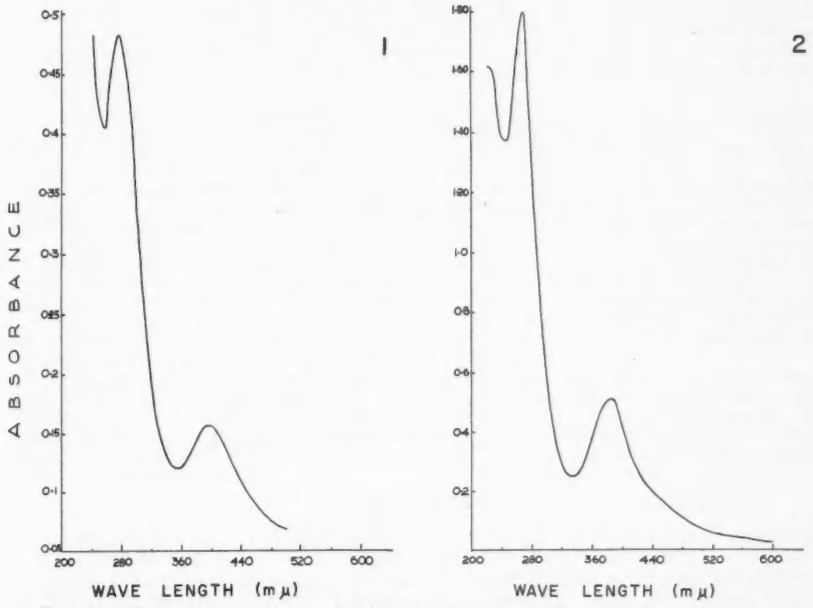

FIG. 1. Absorption spectrum of the bicarbonate-soluble pigment matter from *T. rubrum.*

FIG. 2. Absorption spectrum of the pigment matter extracted with ethylene dichloride from *T. rubrum.*

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